NEUROPHYSIOLOGICAL DEFECTS IN TEMPERATURE-SENSITIVE MUTANTS OF *DROSOPHILA*

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1. INTRODUCTION

Neurophysiological experiments often require blocking of impulses across axons or synapses. This may be done by surgical interruption or by local application of a blocking agent such as a chemical inhibitor or low temperature. Each of these methods has its own disadvantages. Neurosurgical lesions are irreversible and it might be difficult to confine the blocking agent to a specified target, specially within the deep recesses of the central nervous system. A novel method of manipulating biological processes, which has been widely used in molecular genetics, involves the use of temperature-sensitive mutations. Temperature-sensitive mutations result in the formation of a macromolecular species, usually a protein whose tertiary or quaternary configuration is thermolabile. The protein functions normally within a specified range of temperature but, at the 'non-permissive' temperature, it undergoes a change which rapidly blocks the biological activities that critically depend upon its normal configuration. The mutant phenotype is thus expressed conditionally and can be evoked at will by changing temperature. The application of this methodology to the study of nervous system suggests itself as an attractive possibility.

Our own interest in temperature-sensitive mutants of *Drosophila melanogaster* was first aroused by the work of David Suzuki and his associates. Suzuki, Grigliatti and Williamson discovered a mutant of

Drosophila called *para*^{ts} which was instantly paralysed if its temperature was raised to 30°C. When brought back to room temperature, the flies recovered in a few seconds. Subsequently other temperature-sensitive paralysed mutants were found (Suzuki *et al*., 1971; Siddiqi and Benzer, 1972; Grigliatti *et al*., 1973). It was evident that the paralysis of these mutants could be due to a temperature-dependent change in some component of the neuromotor system.

We have isolated a number of paralytic mutations on the X chromosome of *D. melanogaster*. Some of these are alleles of *para*^{ts} and *shi*^{ts}, mutants described by Suzuki and his associates; others belong to new genes *comatose* and TS-2. Electrophysiological tests show that these mutants carry distinctive temperature-dependent lesions in their nerves, muscles or synapses. It is possible, by means of a variety of genetic techniques, to construct mosaics flies only a part of whose body is mutant. The gynandromorphs of *Drosophila* can be made mosaic for genes located on the X chromosome. The expression of the paralysing genes in such mosaics is circumscribed to the male portions of the fly (Suzuki *et al*., 1971). A combination of mosaic technology with mutations affecting components of the nervous system provides an incisive method of analysing the neurophysiological mechanisms of behaviour (Hotta and Benzer, 1972). In the following pages we describe some physiological properties of paralytic mutations on the sex chromosome of *Drosophila* and present results of experiments with mosaics to illustrate the use of paralysed mutants in mapping nerve centres that control motor behaviour.

2. PARALYTIC MUTANTS

Temperature-sensitive paralytic mutations in four different genes on the X chromosome have so far been isolated (fig 1). The mutants *para*^{ts} and *shi*^{ts} have been described by Suzuki and his collaborators (Suzuki *et al*., 1971; Grigliatti *et al*., 1973). Several new alleles of these as well as *comatose* were isolated at Caltech (Siddiqi and Benzer, 1972). Recently Satpal Singh, R.N. Singh and Sheela Donde at Bombay analysed a crop of mutants which includes besides alleles of the previously known genes a new mutant tentatively called *TS2*.

Fig 1 here
Table 1 here

The strains examined and their characteristic properties are listed in table 1. Each mutant is paralysed by a brief exposure to a critical temperature. The paralysing temperatures vary widely between alleles as between different genes. The mutants also differ from each other in their mode of recovery at room temperature. *para*^{ts} and its alleles recover extremely quickly within a few seconds. The recovery of *shi* is also quick but takes somewhat longer than *para*. On the other hand *comatose* recovers very slowly after a lag depending upon the period for which the flies are kept at the non-permissive temperature; after an exposure of 1 minute recovery takes about 5 minutes but when kept at 38°C for 5 minutes it might take 30 minutes or more (Siddiqi and Benzer, unpublished). *TS2* recovers quickly after a brief paralysis at 34°C but, if it is taken above 35°C, the recovery is slow. In all the mutants examined so far, adult flies as well as larvae are affected although the paralysis of larvae might require a different temperature.

3. ELECTROPHYSIOLOGICAL CORRELATES OF PARALYSIS

A few easily performed tests enable one to detect whether impulses in sensory or motor pathways are blocked during paralysis. An electric shock of 2 to 3 volts to the cervical nerve of the fly elicits a jerk in the legs. The response can be monitored either visually or through an electromyogram (EMG) recorded extracellularly from the tibia. The wild type continues to move its legs spontaneously and responds unfailingly to shocks upto 42°C. In $\underline{para}^{ts}$ and its alleles, spontaneous movements of the leg cease but the cervically stimulated response is maintained well above the paralysing temperature. Thus, while some of the pathways in para are blocked others remain operative. Both spontaneous movement and stimulated response are blocked at 38°C in comatose and at 30-32° in $\underline{shi}^{ST-139}$; comatose and shibire thus appear to have a lesion between the excitation of the cervical nerve and the contraction of the muscles in the leg.

The electroretinogram (ERG) of Drosophila, evoked by a short flash of light, contains two prominent components, a corneal positive on-transient and a corneal negative wave (Hotta and Benzer, 1969). The negative wave reflects the depolarisation of the photoreceptor cells which in turn triggers the on-transient, apparently in the second order neurons of the lamina. The ERG of wild type as well as para remain essentially unchanged upto 38°C except for a shortening of latency. In both comatose and $\underline{shi}^{ST-139}$, while the negative wave is not affected, the positive spike is reversibly eliminated by high temperature (Siddiqi and Benzer, 1972). A similar result using long-flash ERG has been

obtained by Kelly and Suzuki (1974) who find that, in shi^{ts}, temperature blocks the on and off transients without affecting the receptor potential.

EMG and ERG are average responses from a large number of cells. In order to obtain more definitive clues to the nature of the block in the mutants, it is useful to make recordings from single cells. The flight muscles provide a convenient preparation for this purpose.

Intracellular recording from flight muscles:

The dorsal longitudinal muscles (DLM) are large fibrillar muscles, six on each side, that indirectly control wing beat by contracting and relaxing the thorax. Each muscle is a single cell, about 120 by 60 μM in cross section, which can be easily impaled with glass microelectrodes. The DLM are innervated by motoneurons in the thoracic ganglion and by the giant neurons whose cell bodies lie in the head. The cervical nerve is a bundle of 3500 fibres including the two giant axons about 10 μM in diameter. There is good anatomical and physiological evidence that the collaterals of each giant axon terminate directly on the DLM of the contralateral side. Other fibers are connected to DLM through one or more interneurons in the thorax (Power, 1948, Tiegs, 1955; Coggshal, et al, 1973; Levine and Hughes, 1973; Levine and Tracey, 1973; Levine, 1974). The recording preparation and the probable circuit of the pathway are shown diagrammatically in fig 2. The resting potential of the muscles ranges from -70 to -85 millivolts and cervical stimulation elicits a single action potential with a small overshoot (McCann and Boettiger, 1961). The delay from the stimulus to the beginning of the action potential is 1.2 milliseconds, consistent with the assumption that the

fibers most readily stimulated in these experiments are the giant axons that terminate directly upon the DLM. The fibrillar muscles of dipterans are multiterminally innervated (Tiegs, 1955). The muscle membrane is electrically excitable like the twitch fibers of verterbrates but the action potential is not an 'all or none' propagated response. It is gradedly dependent on the strength of the depolarising stimulus. The entire fiber fires at once, the magnitude of the recorded action potential being the sum of a large number of local responses (McCann and Boettiger, 1961; Usherwood, 1969). Neuromuscular blocking agents such as ether or tryptamine produce a graded diminution of the response (Hill and Usherwood, 1961; McCann and Reece, 1967). One can, therefore make the following prediction about the possible effect of temperature on the response of DLM in paralytic mutants. If temperature blocks the action potentials in the main trunks of the axons, there should be a sudden, 'all or none' loss of the muscle response. On the other hand if individual terminals of the axons or the neuromuscular junctions are inactivated, one might expect a graded decline in the action potential.

fig 3 here

The effect of temperature on the DLM of wild type is shown in fig 3. As the temperature is raised to 39°C the lag from stimulus to response decreases to 0.8 milliseconds and the time scale of the response contracts making the spike appear sharper, but neither the resting potential, nor the amplitude of the action potential is affected. The pathway from cervical nerve to DLM remains essentially intact.

When the temperature of $\underline{para}^{ST-42}$ or its alleles $\underline{para}^{ts-1}$ and $\underline{para}^{ST-109}$ is raised, there is a sudden 'all or none' failure of the

action potential at a certain critical temperature. If the temperature is reduced below this critical value, the action potential reappears (fig 4). The temperature at which the response fails is somewhat variable from fly to fly and is a few degrees above the paralysing temperature of the strain but, in a given preparation, the responses of all DLM fail at the same temperature. At the point where the response has just failed it can be evoked again by raising the stimulus. The threshold, however, rises very rapidly and, within a minute or so, the preparation becomes inexcitable by a ten times higher stimulus. Upon recovery, the threshold rapidly comes down to the usual 3 volts. In many preparations, a small signal precedes the action potential by half a millisecond. This is, most likely, the presynaptic nerve impulse. When the action potential fails the presynaptic signal also disappears. The 'all or none' failure of the action potential, the increased threshold of excitation and the extinction of the presynaptic signal, all tend to show that temperature affects the excitation of the nerve.

The effect of temperature on $\underline{com}^{ST-53}$ is of a different kind. At 38°C the response undergoes a series of seemingly complex changes. The amplitude of the spike is gradually reduced and a prominent inflexion in the rising phase appears. The action potential falls gradedly to what looks like the junction potential and finally the junction potential itself disappears. Within about a minute at 38°C no traces of neural input into muscle can be seen (fig 5). The recovery at room temperature is also graded and follows the same sequence in reverse. The graded decline and recovery of the action potential in $\underline{com}^{ST-53}$, as against the 'all or none' effect in para, suggests that temperature causes a failure

of individual nerve endings or synapses or both.

The behaviour of shi^{ST-139} is similar to that of comatose except that, in this case, the graded decline in action potential occurs at a lower temperature of 30°C and the muscle action potential is gradually reduced to a small end-plate like potential. At room temperature, the action potential rapidly recovers its normal size and shape (fig 6). The reduced responses may persist for a long time but if the temperature is raised above 32°, it becomes greatly attenuated and recovery takes several minutes. The chief difference between shi^{ST-139} and com^{ST-53} seems to be that, in shi^{ST-139}, although the muscle response is drastically affected, the nerves continue to fire at the non-permissive temperature. Additional evidence is presented in the following sections to show that temperature does not block the propagation of nerve impulses in shibire but affects the neuromuscular junction.

Whereas in the mutants described above, temperature has no effect on the resting potential, the resting potential of DLM^{in}/TS2, rises towards zero above 34°C. On reversing temperature, the cell repolarises, fairly quickly if the exposure to high temperature was short but very slowly if it was kept above 36°C for more than a minute or two. In addition to this the threshold of cervical excitation increases and the ability to follow repetitive stimulation is severely curtailed.

Excitability of muscle membrane:

In order to test whether temperature affects the excitability of the muscle itself, the response of DLM to direct electrical excitation

was examined. A longitudinal flight muscle was impaled with two glass microelectrodes. Through one of these a pulse of depolarising current was injected while the other electrode recorded the response. The response to cervical stimulation was monitored simultaneously. The effect of direct intracellular stimulation on wild type is shown in fig 7. The muscle responds to a 30-millisecond pulse of 200-600 nanoamperes with a repetitive discharge of spike-like potentials. In many preparations the direct response persisted unchanged on repeated stimulation, while, in others, continued current injection reduced the excitability of the muscle. The majority of wild type preparations remained excitable upto 39°C. At high temperatures the response often changed from sharp spikes to a nearly sinusoidal discharge. In some of the preparations the spikes became attenuated and were eventually lost irreversibly, presumably due to damage.

The mutants $\underline{com}^{ST-53}$ and $\underline{para}^{ts}$ behave essentially in the same manner as the wild type. At a temperature where the indirect response to cervical stimulation was lost, the response to direct stimulation clearly persisted (figs8 and 9). The effect of temperature on para and comatose, therefore, cannot be due to inexcitability of the muscle membrane.

In $\underline{shi}^{ST-139}$, the response to direct stimulation, along with the indirect response was greatly reduced above 32° (fig 9). Electrical excitability of the muscle recovered on return to room temperature but not fully, perhaps because of cell damage. The lesion in this mutant, therefore, appears to be postsynaptic as distinct from the presynaptic

defects in comatose and para.

Propagation of impulse in larval nerve:

In order to examine the effect of temperature on the propagation of impulses in nerve axons, it is necessary to record from a pathway in which there are no intervening synapses between the site of stimulation and the site of recording. In the larva of Drosophila eight pairs of abdominal nerves run from the ganglion to the segments of the body (Hertweck, 1931). The exposed nerves can be stimulated at one end and the ensuing action potentials recorded at the other with a suction electrode. Since, in the mutant strains, larvae as well as adult flies are sensitive to temperature, the larval nerves can be used to test the effect of temperature on impulse conduction.

The nerves of the wild type larvae continued to fire at 39°C (fig 10). shi^{ST-139} was indistinguishable from wild type in that impulse propagation survived at 39°C. On the other hand action potentials in $para^{ts}$ were blocked at 32-35° and in com^{ST-53} at 38° (fig 11). These results are consistent with the experiments on flight muscles which indicate a presynaptic lesion in para and comatose and a postsynaptic defect in shibire.

4. TEMPERATURE-INDUCED FLIGHT FIRING

The motor neurons innervating the fibrillar flight muscles of dipterans fire in a regular clockwork fasion. The interspike intervals

are constant and the fibers terminating on the muscles of the same motor unit are phase-locked, that is to say their respective spikes tend to occur in a stable temporal order. The characteristics of neural input into flight muscles are explained by a model which assumes that the neurons driving a motor unit share a common excitatory input and are mutually linked by lateral inhibitory connections. The ensemble of cells generating the patterned firing of flight muscles constitutes the neural flight oscillator (Wilson, 1966; Wyman, 1966 and 1969). The flight oscillator of Drosophila has been analysed by Levine (1973).

As the temperature of shi^{ST-139} approaches 30°C, the DLM begin to fire in the characteristic manner of flight firing. As described earlier, the muscle action potentials were greatly reduced in size but patterned firing continued for many minutes (fig 12). The firing of neural oscillator was not accompanied by wing beats although the wings were free to move.

fig 12 here

It is likely that, in shi^{ST-139}, elements that normally inhibit the oscillator are themselves inhibited at high temperature so that the oscillator runs freely. Patterned generation of reduced junction potentials at the non-permissive temperature provides independent evidence that the propagation of nerve impulse is not blocked and the defect lies at the junction. Incidentally, temperature-induced flight firing provides a convenient method for studying the neural control of flight in solidly tethered flies. Wong (personal communication) has made use of temperature-sensitive mutants for this purpose. Kelly and Suzuki (1974), while recording the ERG of shi^{ts}, observed an oscillatory discharge at elevated temperature which was presumably generated by flight muscles. Induced flight firing

does not occur in wild type, *para* or *comatose*, which is consistent with the finding that, in both *para* and *comatose*, nerve impulses are blocked. On the other hand *TS2* exhibits temperature-induced firing somewhat similar to *shi*$^{ST-139}$.

5. MOSAIC MAPPING OF NERVE CENTRES

Gynandromorphs of *Drosophila* arising from a fertilized egg heterozygous for sex-linked recessive mutations are genetic mosaics, the male portion exhibiting the mutant phenotype. Imagine a mosaic fly carrying a temperature-sensitive paralytic mutation affecting some component of the nervous system. A part of the system can now be switched off by simply raising the temperature. The resulting effect on the behaviour of the fly provides information about the organisation of the system. This is closely akin to making neurosurgical lesions except that the lesions are reversible. The mosaic technique has achieved a high degree of refinement by the introduction of mapping methods originally discovered by Sturtevant many years ago (Sturtevant, 1929; Garcia-Bellido and Merriam, 1969). The fate maps constructed by this method are formal representations of the fly on a two-dimensional surface, each point on the map representing the embryological focus of some adult feature in the blastoderm. Hotta and Benzer (1972) have shown that mosaic mapping can be gainfully used to analyse the embryological origins of complex behavioural traits.

Suzuki, Grigliatti and Williamson (1971) found that in a mosaic carrying *para*^{ts}, the male portion of the fly was paralysed while the

female portion remained active. We have examined the leg movements of several hundred mosaics of $\underline{para}^{ts}$, $\underline{para}^{ST-42}$ and $\underline{para}^{ST-109}$ as well as $\underline{com}^{ST-53}$. In mosaics of $\underline{para}^{ts}$ and $\underline{para}^{ST-42}$, most legs with the mutant phenotype are paralysed while legs with normal cuticular markers remain active at high temperature. At the same time a small proportion of legs exhibit 'recombinant' behaviour, that is to say externally normal legs are paralysed and mutant legs are active. One can, therefore, determine a separate focus for each leg. These foci, shown in fig 13, are located in a region of the fate map which corresponds to the ventral nervous system. $\underline{com}^{ST-53}$ gives similar results. In these three mutants, each leg behaves as a unit during paralysis.

fig 13 here

The result with $\underline{para}^{ST-109}$ is strikingly different. In a given mosaic fly at 35°C, either all legs are paralysed or all legs are active. The focus of leg paralysis is, therefore, a single region somewhere between the thoracic and the cephalic parts of the nervous system. Evidently, the paralysis of $\underline{para}^{ST-109}$ involves a more central element in the circuit controlling leg movement than the paralysis of $\underline{para}^{ts}$ or $\underline{com}^{ST-53}$. The paralytic mutations in conjunction with the mosaic technique thus enable us to distinguish nerve centres controlling complex motor behaviour. The fact that different parts of the nervous system may differ in degree of sensitivity to temperature, allows us to cause selective perturbations in the system and, with the help of mosaics, map out the neural organisation of behaviour.

6. CONCLUSIONS

The mutations described here represent a small sample of possible genetic defects, restricted to the X-chromosome. No doubt many more such mutations will be found. From a closer study of the mutants one might hope to learn something about neurophysiological mechanisms. At present we know very little of the structural causes of paralysing lesions. $\underline{shi}^{ts}$ exhibits an increased tolerance to tetrodotoxin, which, in mosaics, is confined to the mutant tissue (Kelly, 1974). This has led Kelly to conclude that regenerative sodium channel is altered. Our experiments on DLM and larval nerve, on the other hand, show that nerve conduction in $\underline{shi}^{ST-139}$ is unimpaired and the defect is most probably postsynaptic. Other mutants might be defective in potassium, calcium or chloride gates. If so, these mutations could provide us with a way of probing the organisation of the ionic channels. Yet other mutants might be affected in the synthesis, release or degradation of neurotransmitters or in receptor proteins. Such genetically induced perturbations in the machinery of the nervous system would be a useful addition to the armoury of neurophysiological research.

The effect of mutations examined so far appears to spread over the entire nervous system. This is not unexpected. The alteration or loss of a specific membrane molecule, for instance, can, at one stroke, change the function of nerves, synapses or muscles. The observed paralysis of a limb at elevated temperature corresponds to dysfunction of that part of the circuit which fails first. As the system is wired up in a complex fashion, dysfunction of an inhibitory neuron might appear as an excitatory effect elsewhere. The method of mapping is quite general and may, in principle, be used to

analyse any behavioural output that can be selectively triggered by temperature. It should be possible, for instance, to map the nerve centres controlling flight oscillations in much the same way as one can map the foci for leg movements.

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LEGENDS TO FIGURES

Fig 1. Paralytic genes on the X chromosome of *D. melanogaster*.

Fig 2. Set up for recording intracellular responses of longitudinal flight muscles: A. Preparation. B. Diagram of connections from cervical connective to dorsal longitudinal muscles.

Fig 3. Effect of temperature on the DLM response of wild type (CS) flies.

Fig 4. The 'all or none' loss of DLM response in $para^{ST-42}$: Note the disappearance of the presynaptic signal. During paralysis and early recovery in paralytic flies, the muscle is prone to fire more than once (also see fig 6).

Fig 5. The graded loss and recovery of cervically stimulated muscle response in com^{ST-53}.

Fig 6. Effect of temperature on shi^{ST-139}: The reduced junction potentials can be maintained for prolonged periods.

Fig 7. The response of wild type DLM to direct electrical stimulation at different temperatures.

Fig 8. Electrical excitability of $para^{ST-42}$ at different temperatures: The indirect response failed at 37^{o} but the direct response was unaffected.

Fig 9. Effect of temperature on electrical excitability of DLM: A. shi^{ST-13} B. com^{ST-53}.

Fig 10. Effect of temperature on propagation of impulse in larval nerve of wild type:

Fig 11. Effect of temperature on larval nerve of comatose.

Fig 12. Temperature induced flight firing in shi^{ST-139}: A. Flight oscillations begin at 28°C. Patterned firing is maintained even though the muscle response is greatly reduced. B. Cell # 1 and # 2 are phase-locked DLM fibers on the same side. Though the spikes are greatly reduced the pattern of firing is maintained. Multiple sweeps were triggered by the spikes of cell # 1.

Fig 13. Mosaic mapping of paralysing foci with paralytic mutants: The letter symbols denote different cuticular markers (see Benzer this volume), the numbers show map distances between foci.

Table 1

Paralytic behaviour of some temperature-sensitive strains*

Strain	Paralysing temp.	Recovery
WILD TYPE (CS)	42°	
$para^{ts-1}$	29°	VERY QUICK (15 sec)
$para^{ST-42}$	30°	
$para^{ST-109}$	32-34°	
$para^{TS-3}$	34-36°	
$para^{TS-11}$	28°	
shi^{ST-139}	30°	MODERATELY QUICK (30 sec - 2 min)
shi^{TS-1}	37°	
shi^{TS-4}	34-36°	
shi^{TS-6}	27°	
com^{ST-53}	38°	SLOW (5 to 60 min depending on heating
com^{TS-7}	38°	
TS-2	34°	QUICK OR SLOW (depending on heating)

*The strains carrying the superscript ts are from Dr. Suzuki's laboratory. Strains designated ST were isolated at Caltech and those designated TS at Bombay.

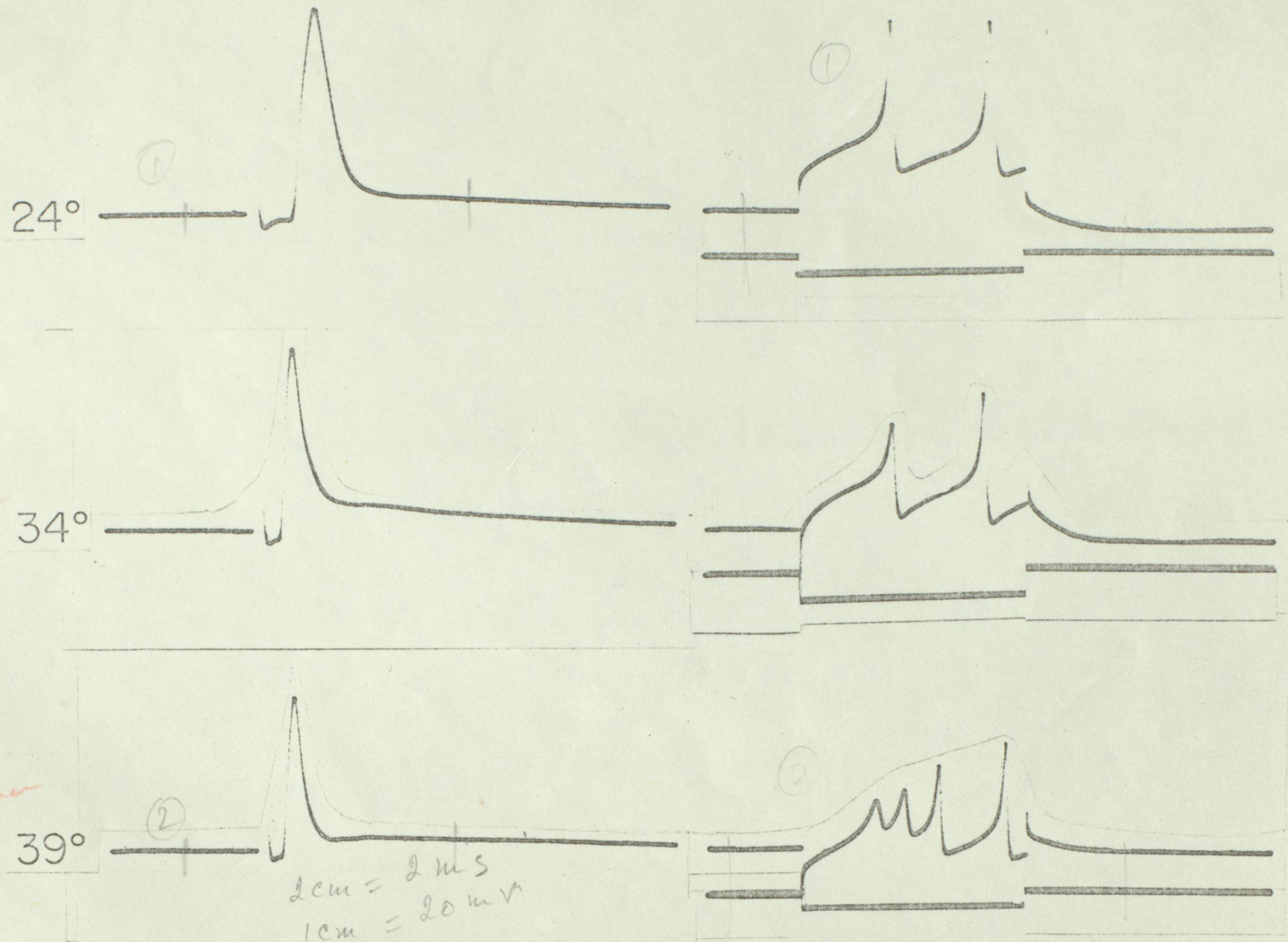
1 to do -

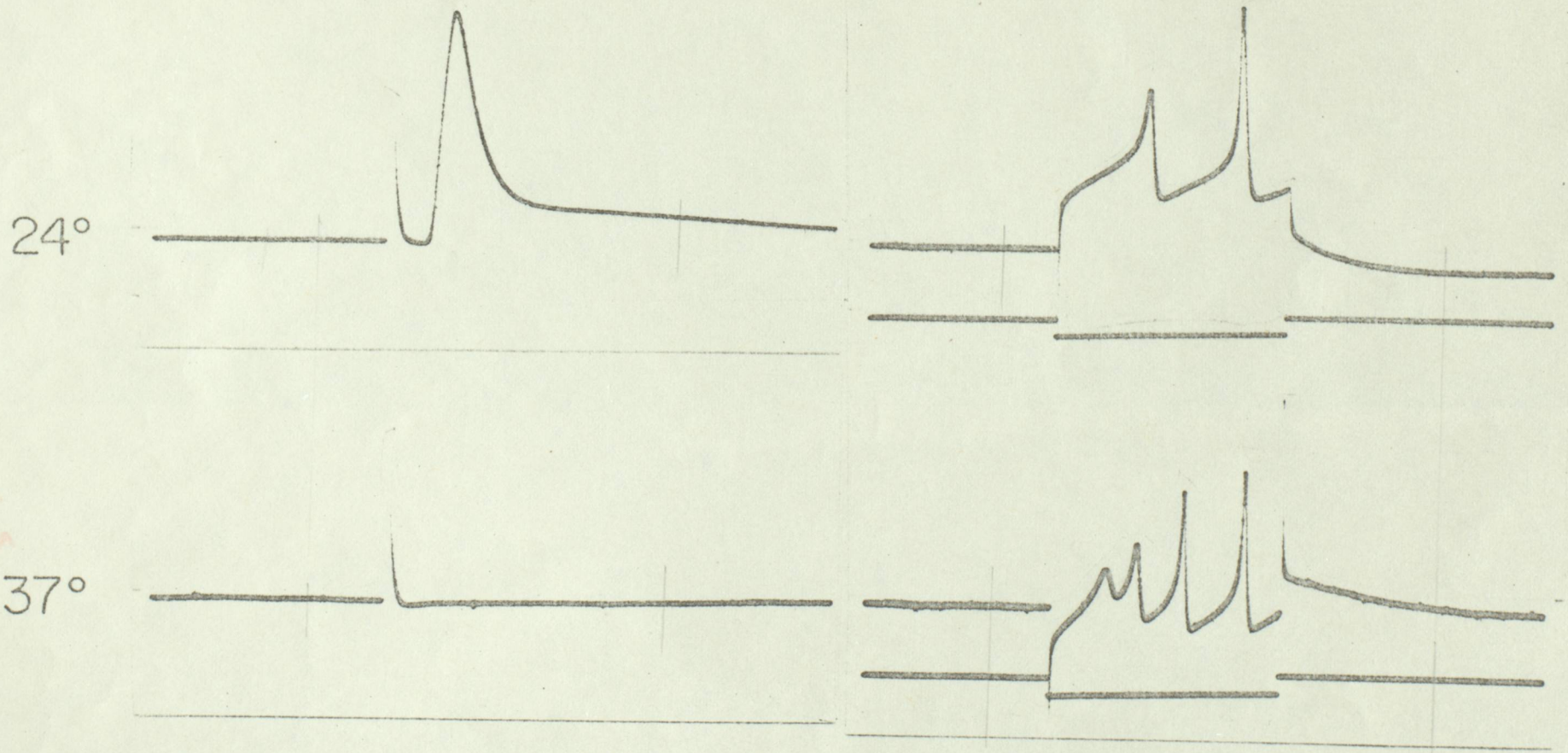
1. Make larval records | combined records for
STGL
hairs - | CS. com. shi. & r

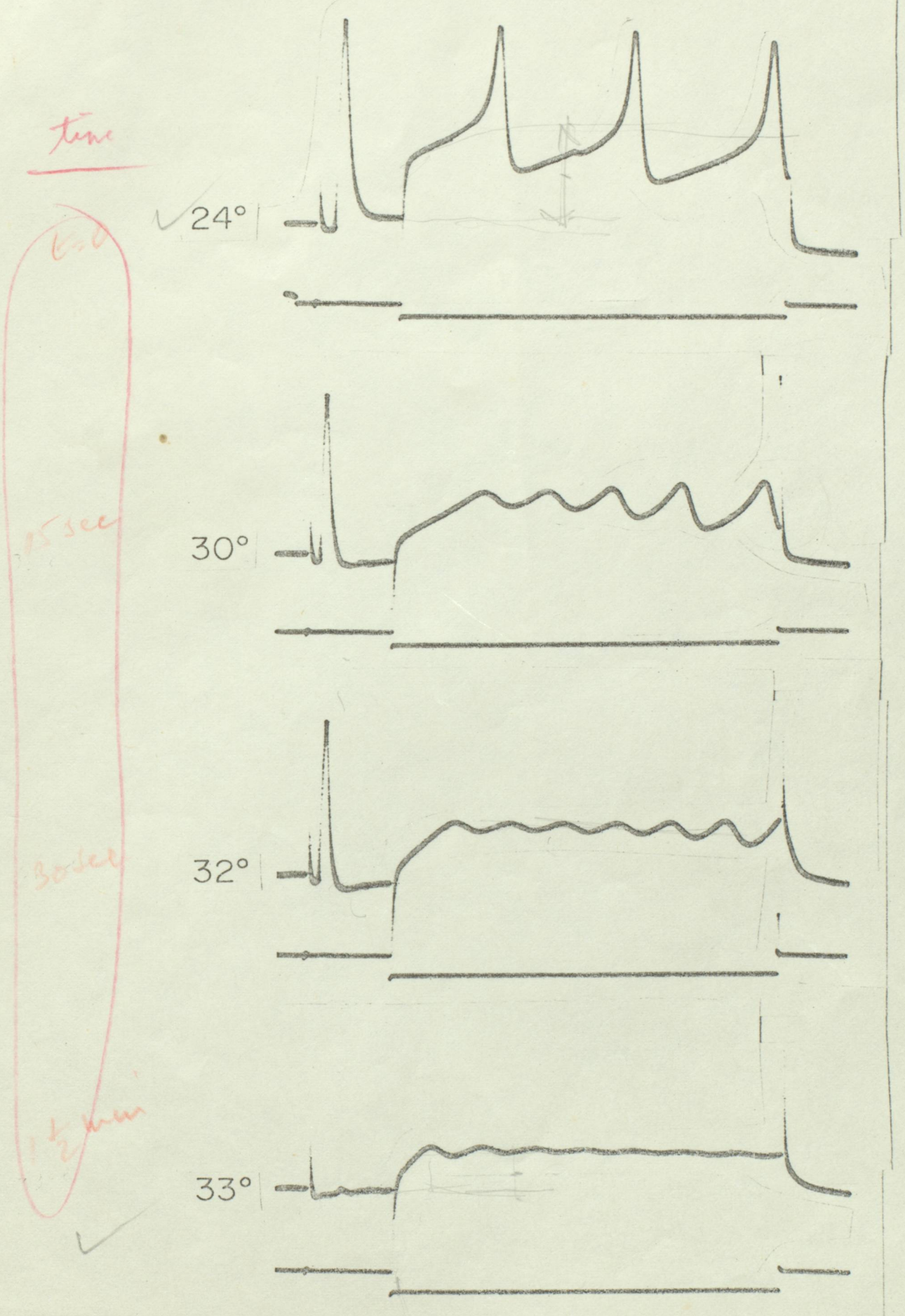
2. Make combined ERG. pictures -
CS, com., shibuni & hairs;

(3)

4. Repeat shibuni flight muscle
experiments - (Repeat).


24°
34°
39°
2cm = 2 mS
1cm = 20 mV
CERVICAL STIMULATION
DIRECT STIMULATION


24°
37°
CERVICAL STIMULATION
DIRECT STIMULATION


time
24°
30°
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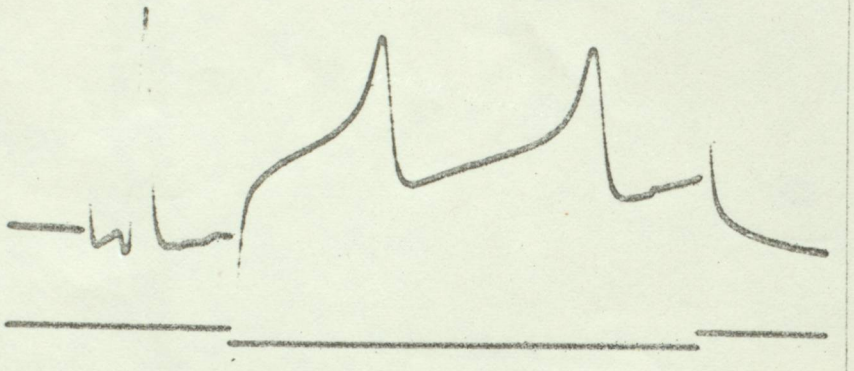
ST139

±

0

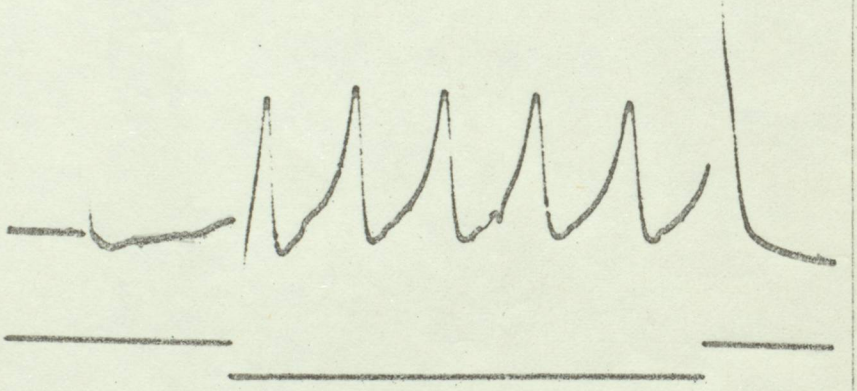
24°



✓

1.5 min

38°



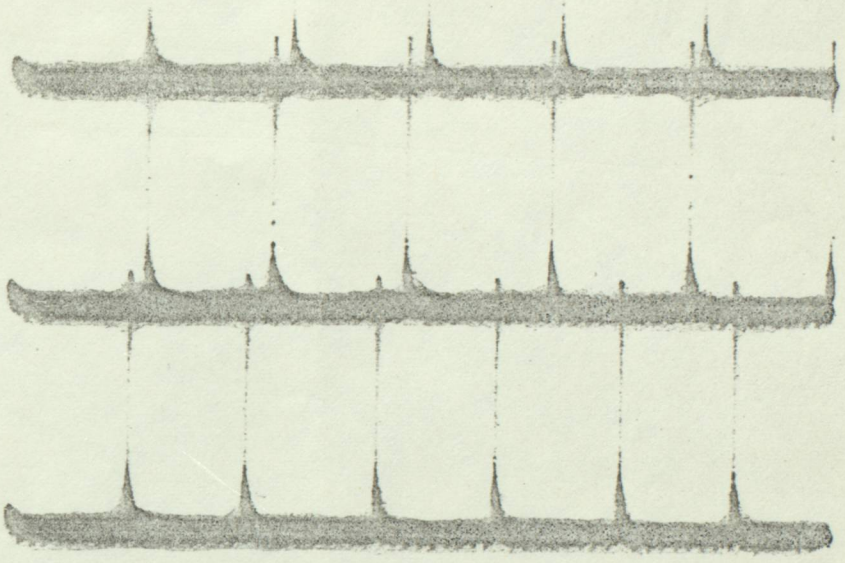
contre

t = 0, 24°

28°

(t=?)

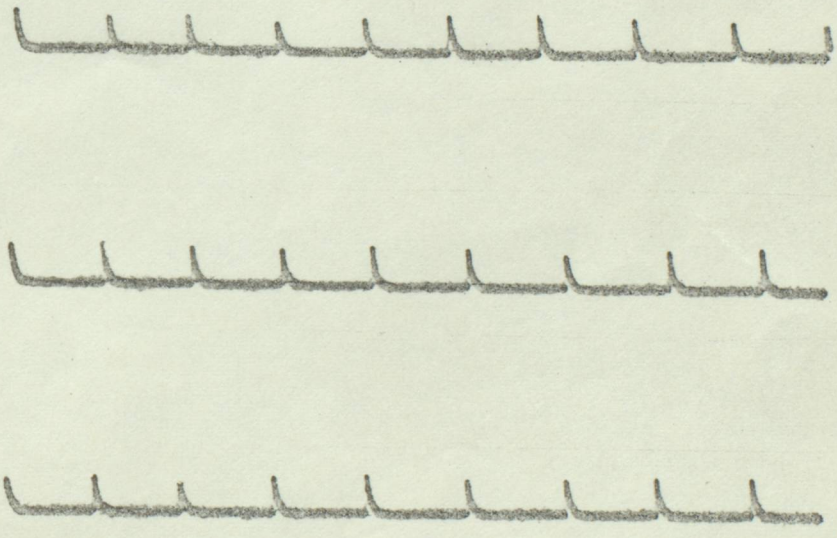
30 sec



32°

(t=?)

2 min



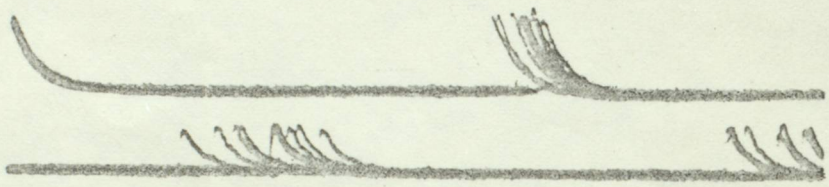
ST139

320

t = 4 min

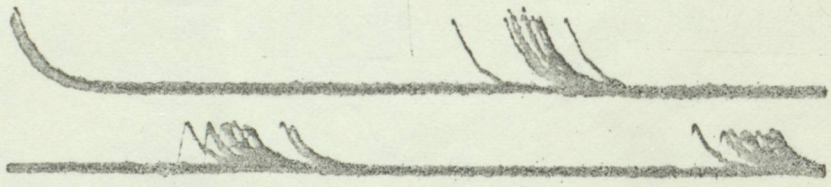
Cell #1

#2



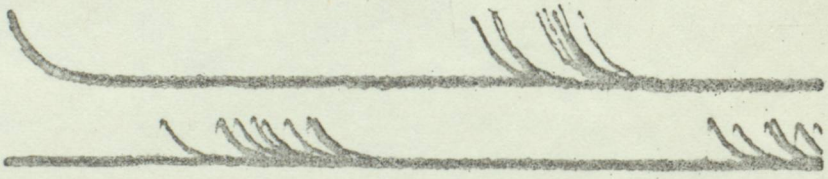
Cell #1

#2



Cell #1

#2

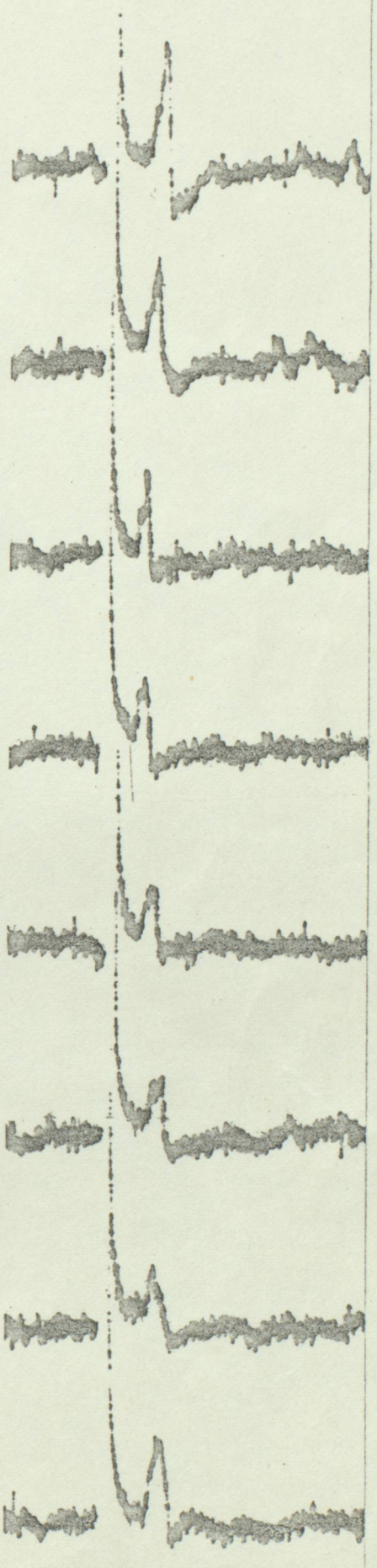


STBY

t	T_{ear}
0	25.6
0.5 min	36
1	39
2.5	39.9
3 min	37
3½ 7 min	30
4 min	28
5.5 min	27

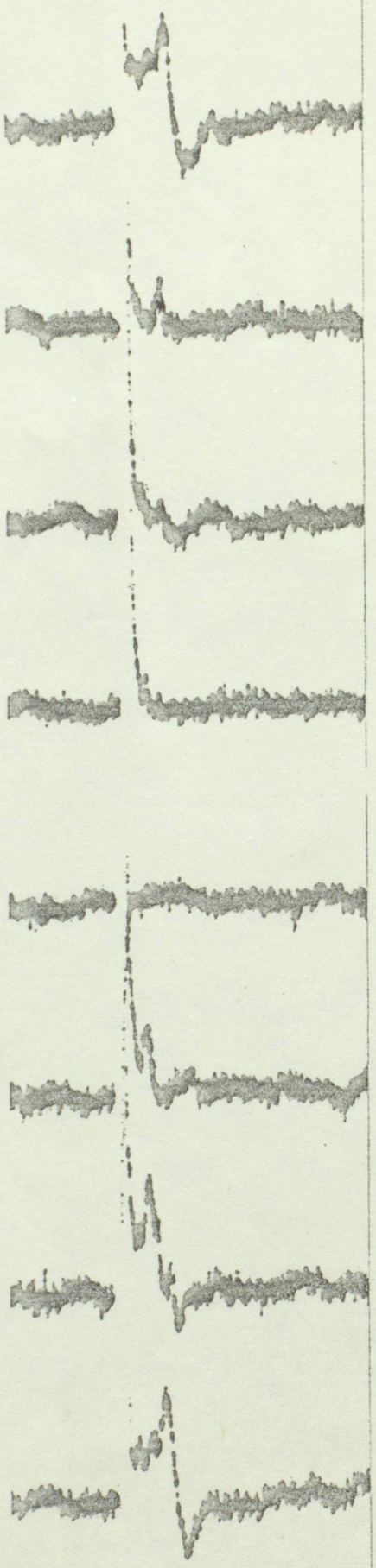
Heat ↑

Approx.



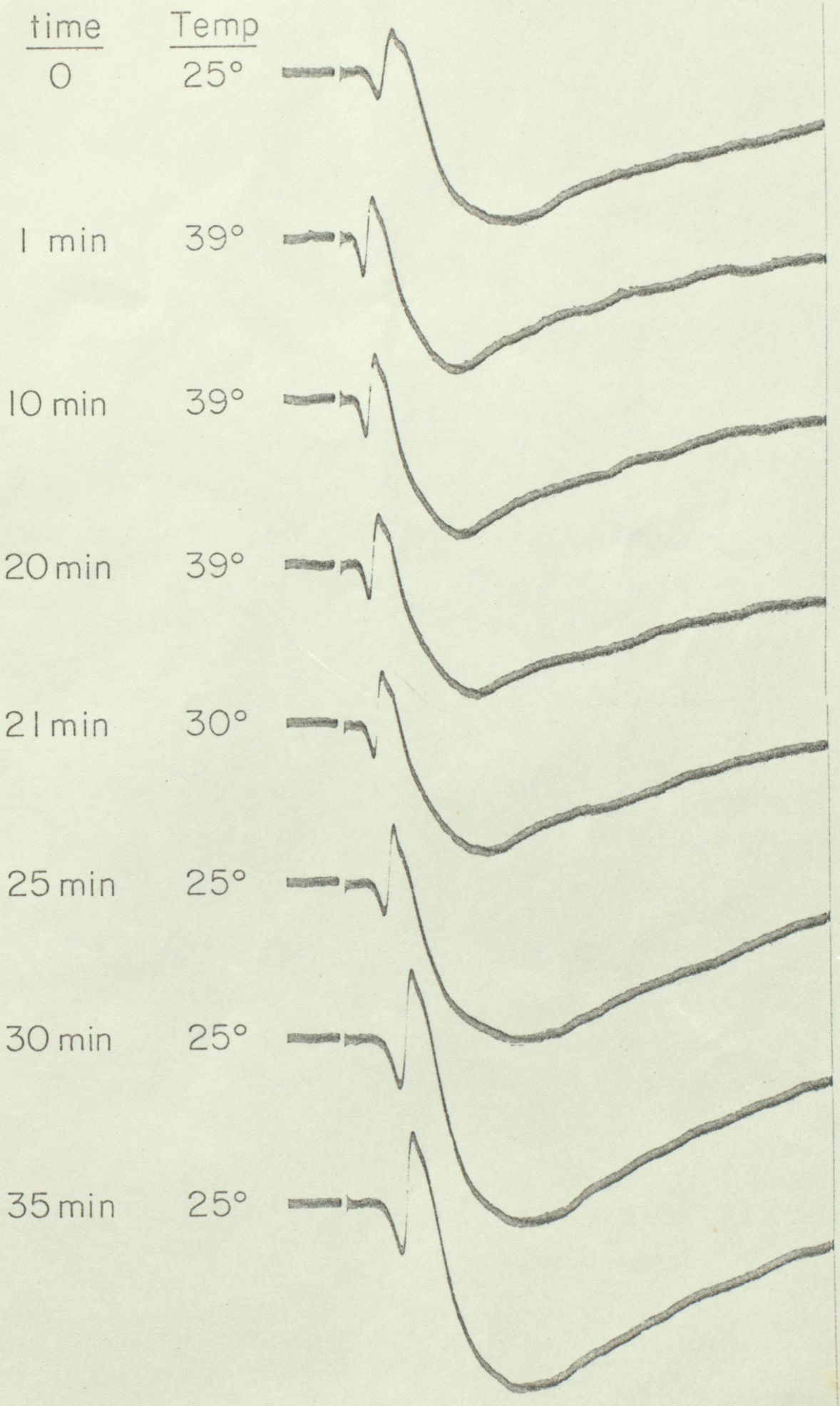
Approx

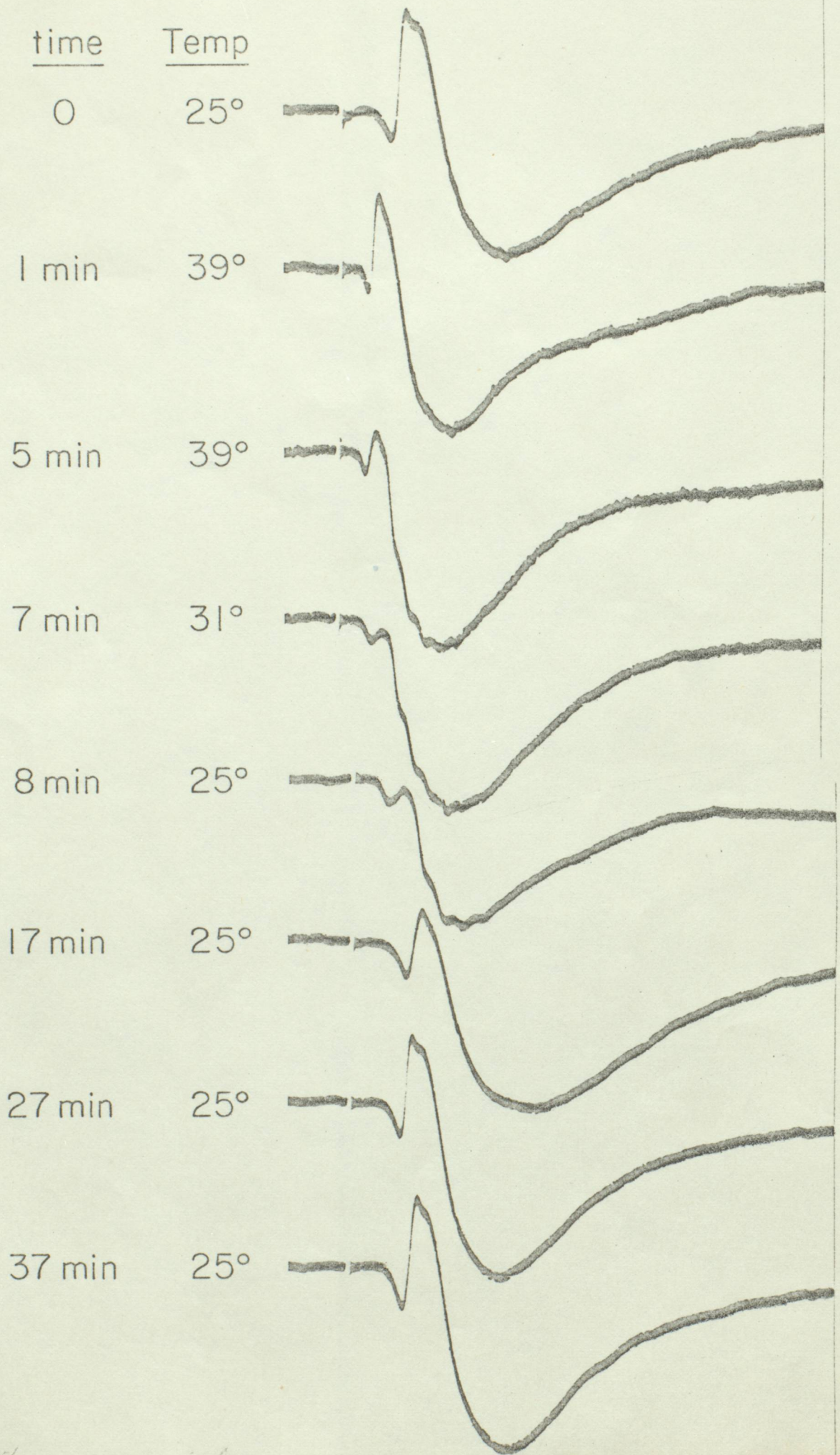
Time	Temp
0	26.4
30 sec	37.5?
45 sec	38.0
1 min (after heat off)	38.5
2 — 3 min	34
3 — 4 min	32
5 — 5 min	29
8 min	26.8



Absolute times needed, starting with t=0 at top

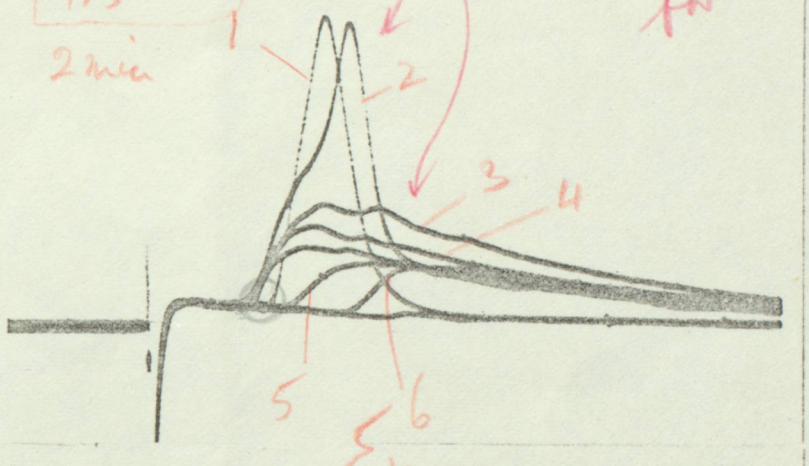
Contour


time
Temp
0
25°
1 min
39°
10 min
39°
20 min
39°
21 min
30°
25 min
25°
30 min
25°
35 min
25°

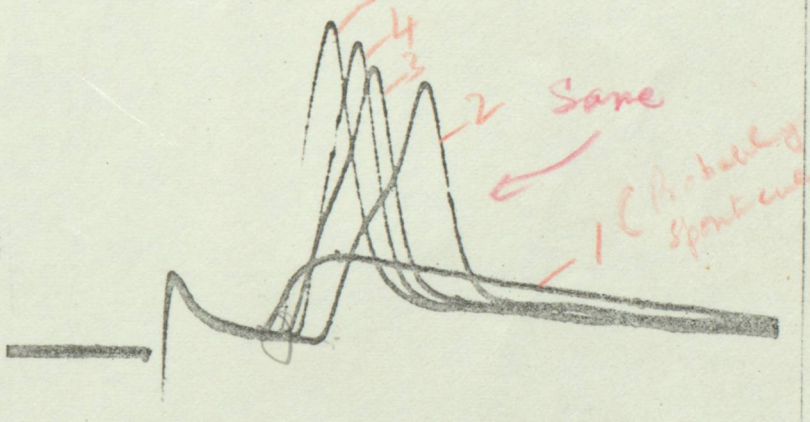

time
Temp
0
25°
1 min
39°
5 min
39°
7 min
31°
8 min
25°
17 min
25°
27 min
25°
37 min
25°

	temp	time
1	24°	0
2	30	20 sec
3	30.5	40 sec
4	31	60 sec
5	32°	1.5 min
6	32°	2 min

Approximately
Later:
number the traces in ~~degrees~~
and tell me
time and temp
for each.



RISING TEMP.
(to 31°C)



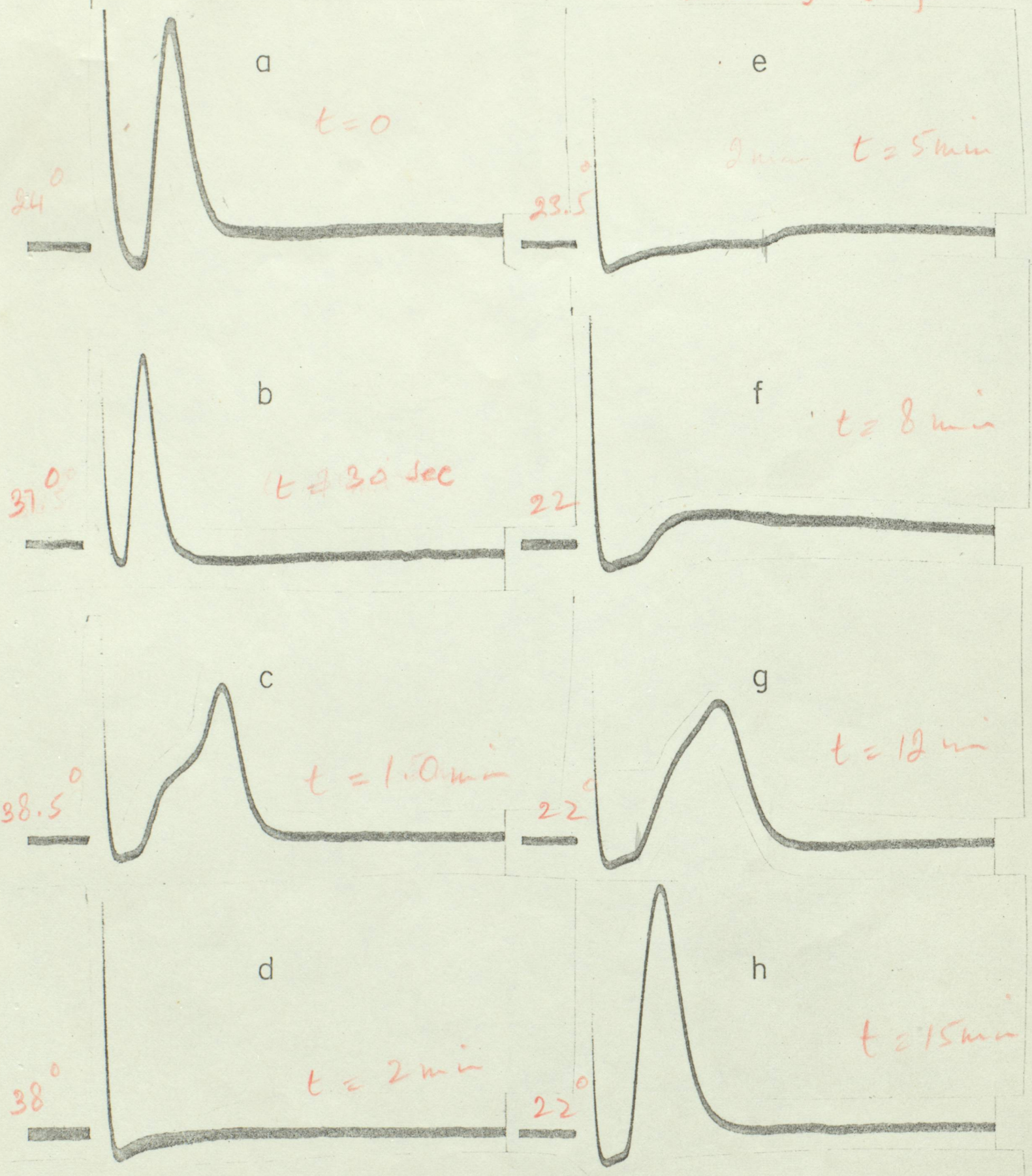
FALLING TEMP.
(to 22°C)

	recovery temp	time
1	25°	3 min (from beginning)
2	23	—
3	22.5	
4	22	—
5	22	5 min

Approx

ST13 #6

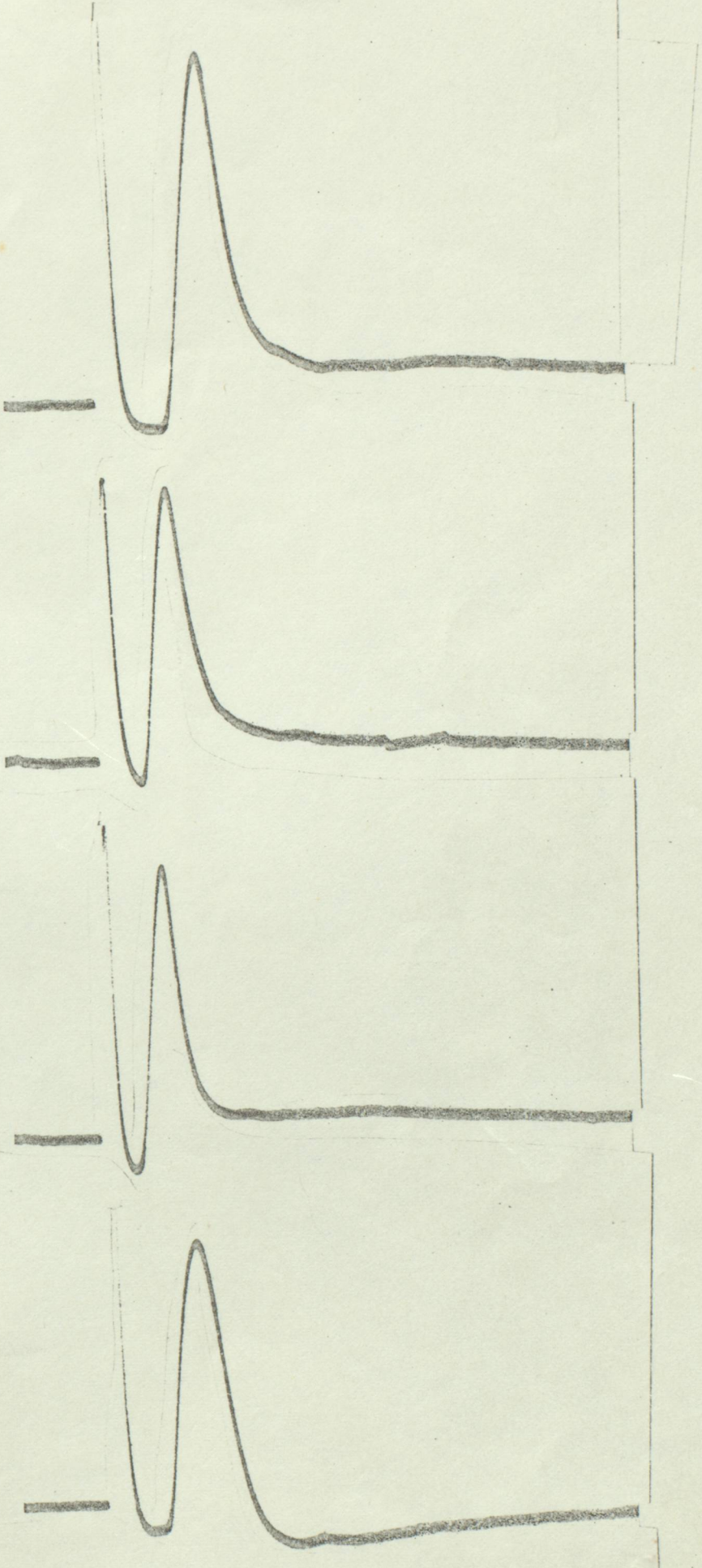
Recovery e-f:



Constore (?) Data on time ad temperture for each ?

(old exp time in

Time	Temp °C
0	25°
1.5 min	38°
3 min	39°
8 min	25°



Neurophysiological Defects in *comatose* and other Temperature-Sensitive Paralytic Mutants of *Drosophila melanogaster*

INTRODUCTION

Suzuki, Grigliatti and Williamson (1971) described a mutant gene *paralysed*, on the X-chromosome of *Drosophila* that causes instant paralysis at temperatures above 30°C. When the temperature is reduced the fly recovers quickly. Another mutant *shibire*^{ts} behaves similarly (Grigliatti et.al., 1973). Paralytic mutations could affect different parts of the nervous system. In certain mutants, paralysis might be due to a failure of axonal conduction; in others transmission at a particular kind of synapse might be blocked; yet others might be defective in the neuromuscular junction or the muscles. These defects could, for instance, emanate from mutations in genes responsible for the synthesis of membrane components or transmitter molecules. Mutations of this kind would provide a potentially powerful tool for analysing the neuromotor system since the defect can be turned on and off at will and the same animal serves as its own control. In addition one can use genetic techniques to construct mosaic flies in which some parts of the animal are mutant and others normal, thus localising the focus of the lesion.

We have isolated several temperature-sensitive mutants and investigated the physiological basis of paralysis in normal, mutant and mosaic flies. Some of our isolates are alleles of *para* and *shibire*, others belong to a new temperature-sensitive paralytic gene. The new

gene comatose, is located at 40 units on the X-chromosome. The comatose flies become paralysed after a brief exposure to 38°C. Whereas the recovery of para^{ts} and shi^{ts} from the same treatment occurs in a matter of seconds, recovery of comatose requires minutes or hours, depending upon the length of exposure to high temperature. In normal flies paralysis does not set in until 42°C and recovery is quick.

Neurophysiological studies reveal distinctions between different paralytic mutants. When the flies are fixed on their backs in wax, the legs show persistent spontaneous movements at room temperature. An electric shock to the cervical chord evokes a sharp jerk in the legs. At the paralysing temperature of 30°C, para stops spontaneous leg movements but when cervical stimulation is applied, the legs still respond.

The result of a similar experiment with the mutant comatose is different. As the fly is raised to 38°C, spontaneous leg movement ceases, but so do the leg jerks in response to cervical stimulation. This defect persists on return to room temperature until the period required for recovery from paralysis is over; comatose appears to develop, in the paralysed state, a block somewhere between the cervical connective and the muscles. The experiments described here were done to localise and identify this block.

The mutant shi^{ST-139}, isolated by us, also stops leg movements, and loses the leg response to cervical stimulation above its temperature of paralysis. Electrophysiological analysis, however, shows its properties to be different from comatose. Thus, mutations in all three temperature-sensitive paralytic genes affect the neurological mechanisms in distinct ways.

MATERILAS AND METHODS

1. Isolation of mutants

Males of the strain Canton-Special (CS) of Drosophila melanogaster were mutagenised with ethyl methyl sulfonate and mated to attach-X females marked with yellow and forked according to the procedure described by Lewis and Bacher (1966). Each F_1 male was again mated to a virgin attached-X female to produce a set of F_2 males carrying identical mutagenised X-chromosomes. Adults of each such line were exposed to 38°C to detect temperature-induced changes in behaviour. Among lines screened, mutants were obtained that became sluggish or paralysed at the elevated temperature. Seven of the mutants are described in this report.

2. Genetic mapping and complementation tests

Female heterozygotes and homozygotes were constructed for all combinations of the seven mutants and tested by exposure to 40°C for one minute. This exposure was sufficient to induce paralysis in all the mutants whether hemizygous males or homozygous females but not in normal CS flies, nor in any of the mutant/+ heterozygotes. By this test the seven mutants could be separated into three complementation groups.

Through the courtesy of Dr. David Suzuki, samples of $para^{ts-1}$ and shi^{ts-2} were obtained and tested against our mutants, revealing that two of our groups coincided with his. The third group comatose, which requires a higher temperature for paralysis, represents a new gene. This gene was mapped by recombination against the markers and on the X-chromosome at position 40 ±.

3. Heating chamber for neurophysiological experiments

The fly was mounted in dental wax on a 0.5 cm cork fixed at the centre of a perspex block (Fig.) surrounded by a metallic shield made of tin can, 3 cm x 4 cm x 3 cm in size and covered at the top with a bakelite lid. The lid had a perspex window for illumination and observation and openings on both sides for placing electrodes. The insulated shield was wrapped with a coil of tungsten wire. The current through the coil was supplied by a Versa-Therm proportional temperature device controlled by a thermister close to the fly. The temperature was measured by a second thermister (YSI No. 427). About 3 minutes were required to raise the heating chamber from room temperature to 38°C. When it was necessary to attain the paralysing temperature quickly, the chamber was prewarmed to the required temperature before inserting the mounted fly.

Subsequently the heating chamber was replaced by a simpler apparatus consisting of a bakelite sheet with a cylindrical cavity of dia. 2 cm and height 0.5 cm, mounted on a glass slide. The floor of the cavity was lined with a thermofoil heating element (Minco Products. Inc. Minneapolis). The fly was mounted in the centre of the cavity and the heating current through the thermofoil was regulated by a variable resistor. The temperature was monitored by a thermocouple microprobe (Cole Parmer No. 8506-30) placed under the abdomen of the fly. The heat capacity of this system was small so that the temperature could be raised or lowered within a minute or so.

4. Cervical stimulation for leg jerk

The fly was mounted ventral side up and the tips of the wings, the abdomen, and the front legs were waxed down. The head was fixed in

wax so as to slightly stretch the neck. Electrolytically sharpened stainless steel needles were used as stimulating electrodes. These were inserted on both sides of the cervical nerve and 2-3 volt pulses of 0.4 millisecond duration were delivered. The normal fly responded to each pulse with a strong jerk of legs.

5. Electromyograms

The response of the leg muscles could also be monitored electrically. The fly was mounted on its back and the legs on one side were stretched and waxed down at the tip. A micropipette filled with 1 molar NaCl was inserted in the tibia of the mesothoraccic leg. A second micropipette placed in the abdomen served as the reference electrode.

6. Intracellular recording from flight muscles

The dorsal longitudinal flight muscles of Drosophila can be easily impaled with glass microelectrodes filled with 3M KCl using the stereotaxic map of the muscle fibers constructed by Levine and Hughes (1973). The fly was mounted, dorsal side up, and its legs were stretched out and fixed. One side of the thorax was supported on a wax stilt taking care that the thoraccic spiracles remained open. The cuticular terminations of the individual muscle fibers can be located with the help of morphological landmarks on the surface of the thorax (Levine and Hughes, 1973). One or more holes were made in the cuticle with a sharpened needle through which the tip of the microelectrode could be inserted in a desired direction. In most of the preparations we could record from each of the six dorsal longitudinal fibers on one side, one after another. The identity of the fiber could, therefore, be established with sufficient confidence. The

reference electrode, a micropipette filled with NaCl, was placed in the thorax. The resting potential of the longitudinal muscles was typically around -70 to -80 mV and the action potentials observed in response to a 2-3 volt stimulus in the cervical chord were similar to those described by McCann and Boettiger (1961) for dipterans.

7. Direct intracellular stimulation of flight muscle

For direct excitation of flight muscles two separate micropipettes filled with 3M KCl, one for current injection and the other for recording, were inserted in the same fiber. A 30 millisecond pulse of 200-600 nanoamperes through the stimulating pipette was usually sufficient in normal flies to elicit a response.

8. Electroretinogram

The ERG in response to a 20 sec strobe flash was recorded as described by Hotta and Benzer (1969). The flash was introduced into the heating chamber with a fiber light guide.

RESULTS

Some of the mutants isolated by us are allelic to $para^{ts}$ and shi^{ts}, mutants described by Suzuki and his associates (Suzuki et.al., 1971; Grigliatti et.al., 1973); others belong to a new gene comatose. The location of the paralytic mutants on the genetic map of the X-chromosome, their allelic relationship and phenotypic characteristics are summarised in table I.

The new gene comatose maps at 40 ± units. The three com alleles are paralysed at 38°C but differ in the duration of paralysis

at room temperature after a given exposure to high temperature. The kinetics of recovery of the adult com flies are shown in Fig. . The mutations are recessive and com/+ heterozygotes are not paralysed. in complementation tests, all combinations of the three mutants alles gave the mutant genotype. Out of these com^{ST-53} was selected for detailed study.

The three new alleles of the previously-described gene para^{ts} have properties qualitatively similar but differ in the temperature required for paralysis. All are recessive and do not complement each other or para^{ts}. Our mutant shi^{ST-139} is an allele of shi^{ts} (Grigliatti et.al., 1973) and has similar properties.

In all of the above mutants locomotor paralysis found in adult flies is also observed in larvae, although a somewhat higher temperature may be required. Thus larvae of para^{ts} become immobilised at 32°C and recover rapidly when the temperature is lowered. shi^{ST-139} behaves the same although recovery is slower after prolonged paralysis. The larvae of comatose like the adults, are immobilised by 38°C or more and remain paralysed for a long time at room temperature. The fact that the larvae show behaviour similar to the adult is useful as the larval nerves offer a convenient preparation to study the conduction of action potentials.

Neurophysiology

We have carried out a number of electrophysiological experiments on normal and mutant flies to determine whether any part of the nervous

or muscular system is affected when a mutant becomes paralysed.

1. Response to cervical stimulation: An easily performed test is to apply a mild electric shock to the cervical nerve and observe the response of the legs. Wild type flies responded to a 0.2 millisecond stimulus of 2 to 4 volts with a strong jerk of all the legs. As the temperature was raised the response persisted upto 40°C for several minutes.

The mutant comatose after one or two minutes at 38°C, did not show leg jerks in response to cervical stimulation even after return to room temperature. A ten-fold increase in stimulus to as high as 20 volts failed to cause any perceptible leg movement. After the requisite recovery period, however, the jerk response returned to normal. Flies exposed to 38°C for one minute recovered fully in about 5 minutes; a 5 minute exposure required more than half an hour for recovery.

The mutant shi^{ST-139} was affected in the same way as comatose, but at a lower temperature. The leg response stopped in about 2 minutes at 32°C, recovering within a minute or two, after return to room temperature. Longer exposures at 32°C - 34°C required longer periods for recovery.

Thus, in both comatose and shi^{ST-139} one or more of the steps along the pathway from cervical nerve to leg muscle are blocked.

In contrast, the para mutants $para^{ts}$, ST42 and ST109 continued to exhibit cervically stimulated leg jerk well above their paralysis temperatures. The paralysis shown by these mutants cannot be due to a generalised failure of nerve conduction, neuromuscular junction or muscle excitability. Something must be blocked, since the flies legs cease spontaneous movement at high temperature, but the experiment either overrides or bypasses that block.

2. Intracellular recordings from flight muscles

To test whether impulses from the cervical nerve actually arrive at muscles, a convenient station to record from is the set of very large indirect flight muscles in the thorax. Each of these is a single cell and can be readily impaled with a glass microelectrode. Intracellular recordings were made from the dorsal longitudinal muscles using micropipettes filled with 3M KCl. The resting potential in the normal flies ranged from -70 to -80 millivolt, and cervical stimulation evoked a single action potential with a small overshoot (McCann and Boettiger, 1961). An example is shown in Fig . The delay from the stimulus to the beginning of the action potential was 1.2 milliseconds. At 39°C the response time decreased to about 0.7 milliseconds and the spike became sharper but the resting potential and the amplitude of the action potential were undiminished for several minutes. Thus, in wild type flies, the entire pathway from cervical connective to flight muscle action potential remains intact at high temperature.

A different result was obtained for the para group of mutants $para^{ts}$, $para^{ST-42}$ and $para^{ST-109}$. When these were taken above 32°C keeping the stimulus pulse constant at about 3 volts, the muscle action potential suddenly dropped to zero at a certain critical temperature. When the temperature was lowered slightly, the action potential reappeared. This was an all or none effect that could be repeated many times by shifting the temperature above or below the critical point. All the longitudinal muscle fibers in a given fly ceased to fire at the same temperature; in different preparations, however the critical temperature varied. The histograms in Fig show the variability in the three mutant strains. The effect is usually discernible only at temperatures well above the

temperature which causes locomotor paralysis. *para*$^{ST-109}$ flies require a higher temperature for paralysis than *para*^{ts} and *para*$^{ST-42}$.

At the point where the muscle response in *para* mutants first fails, it can be evoked again by raising the stimulus by a few volts. But the stimulus threshold rises very rapidly with time and the preparation becomes inexcitable by stimuli exceeding 10 volts. During recovery the earliest response is evoked by a large shock exceeding 10 volts but the threshold quickly comes down to the usual 3 volts. In some preparations a small signal can be seen which precedes the muscle action potential by about 0.5 milliseconds. This may be the presynaptic nerve spike; it disappears, or becomes greatly diminished, *pari passu* with the muscle action potential (Fig). The all or none failure of the muscle response and the increase in the threshold of excitation indicate that in these mutants, temperature affects the excitability of the nerve rather than muscle.

The effect of temperature on the flight muscle response of *comatose* was of a different kind. At 38°C the action potential underwent a reduction in amplitude and an increase in latency. The action potential first changed to a form that looked like a junction potential; that is to say it lost its inflection, and finally, in about 30 seconds disappeared altogether. Recovery at room temperature was slow and graded, showing a similar progression in reverse (Fig.). At the height of paralysis the presynaptic nerve spike" could not be seen; all traces of neural input into muscle had disappeared. The graded effect in *comatose* is distinct from the all or none loss of action potential in the *para* mutants.

The behaviour of *shibire*$^{ST-139}$ resembles that of *comatose* in

showing a graded effect. At 30°C the muscle action potential was gradually reduced to a small end-plate-like potential. At room temperature the action potential rapidly recovered its normal size and shape (Fig.). shibire^{ST-139} is affected at a much lower temperature than comatose and recovers faster. Also the reduced action potential may persist for a long period at 32°C. If the temperature is raised to 34°C or more, the response becomes imperceptible and recovery might take several minutes.

3. Electrical excitability of muscle

Since the flight muscle action potential in response to stimulation of cervical nerve is affected in some of the mutants at high temperature, the question arises whether muscle function itself is intact. The excitability of the flight muscles can be tested by direct injection of depolarising current. A longitudinal muscle fibre was impaled simultaneously with two KCl-filled micropipettes, one of which recorded the intracellular potential while the other was used to inject a 30 millisecond pulse of 200 to 600 nanoamperes of current, using bridge balance. The indirect response of the muscle to stimulation of the cervical nerve was monitored in the same preparation.

In normal flies, the response of the muscle to direct intracellular stimulation is a repetitive discharge of spike-like action potentials (Fig.). This response is somewhat variable; in many preparations the direct response persists unchanged on prolonged repetitive stimulation, while in others, repeated current injection reduces the excitability of the muscle. A majority of the preparations of normal flies remained excitable upto 39°C. At higher temperatures the response changed from sharp spike-like firing to a sinusoidal discharge and in about half of the preparations the spikes

became attenuated and were eventually lost irreversibly, presumably due to damage by high doses of current.

comatose^{ST-53}, para^{ts} and para^{ST-42} behaved at high temperatures essentially in the same manner as the wild type. Under conditions where the indirect response to cervical stimulation was lost at high temperature, the response to direct stimulation was not affected (Fig. and). In the mutants comatose and para therefore, the electrical excitability of the flight muscle itself is not responsible for paralysis.

In the case of shibire^{ST-139} on the other hand, the response to direct stimulation was greatly reduced along with the indirect response at temperatures above 30°C (Fig.). Electrical excitability of the muscle recovered on return to room temperature, although never fully. In shibire, therefore, there appears to be a temperature-sensitive lesion in the muscle itself.

4. Propagation of action potential in nerve

The propagation of action potential in nerves can be recorded extracellularly from a readily accessible nerve in the larva. Since, in these temperature-sensitive mutants, larvae as well as adults are paralysed at high temperature, larval nerves offer a convenient preparation for testing the effect of temperature on impulse conduction.

In the larva, eight pairs of abdominal nerves run from the ganglion to the abdominal segments (Fig.). Third instar larvae were dissected under Drosophila ringer and their nervous system exposed. One of the large nerves, the 7th or the 8th, was cut near the posterior end and sucked up in a micropipette with a tip diameter of 10 meter filled

with Drosophila ringer which served as the recording electrode. The stimulus was applied near the anterior end with a pair of ringer-filled micropipettes touching the nerve. With stimuli just above threshold, the response consisted of a single action potential of about 200 microvolts which persisted upon repetitive stimulation. As the stimulus amplitude was raised, a second, slightly delayed response was observed. The second response failed frequently when the repetition rate exceeded about 20 per second. It is likely that the first response was the result of direct stimulation of the nerve while the later response was indirect, resulting from antidromic excitation of the ganglion.

The nerves of normal larvae continued to fire above 39°C but comatose lost its response at 38°C. When brought back to room temperature, the impulses reappeared after a while (Fig.). Thus there is an effect of temperature directly on nerve excitation in this mutant. This is not the case with shibire^{ST-139} for the nerves of shibire larvae were indistinguishable from wild type, giving conducted action potentials at 39°C for several minutes. The larvae of the para group have not been examined so far.

5. The electroretinogram

The electroretinogram (ERG) of Drosophila, when evoked by a short flash of light, contains two major components, a corneal-positive spike and a negative wave (Hotta and Benzer, 1959). The negative wave is the primary response reflecting depolarisation of the photoreceptor cells. This triggers off the positive spike, which is believed to arise from the action potentials of the second order neurons in the lamina. The ERG of normal flies remains basically the same at temperatures up to 38°C, except for a contraction in the time scale. The same is true of

para^{ts}; heating the fly above its paralysing temperature does not change its ERG (Suzuki, et.al., 1971). In *comatose*, at 38°C the photoreceptor response remains intact, but the positive peak is lost. On returning to room temperature the ERG gradually recovers its normal form (Fig.). This effect of high temperature on the ERG is not as rapid as the paralysis of the motor system and is somewhat variable among individual flies as shown in Fig. . The same behaviour is shown by *shibire*$^{ST-139}$ except that the positive peak is lost at a lower temperature of about 32°C and recovery is quicker, usually taking no more than one or two minutes. This is the same as reported by Hall ( ) and Kelly (1974) for *shibire*. Hall has shown that, in genetically mosaic flies, it is the genotype of lamina and not the retina, that determines whether positive spike will be lost at high temperature.

Thus, in all three kinds of temperature-sensitive paralytic mutants, photoreceptor cell function is resistant to the temperature which causes paralysis. Synaptic transmission from the photoreceptors to the second order neurons in the lamina and excitability of the lamina must ramain intact in *para*, while in *comatose* and *shibire*, one or both of these processes break down at high temperature.

6. Temperature-induced firing of flight oscillator

The ensemble of indirect flight muscles in *Drosophila* and related dipterans is driven by neurons that fire in a characteristic pattern. The motor neurons innervating the different muscle fibers of a motor unit fire at close to the same frequency, maintaining fairly constant interspike intervals with stable phase relationships between different fibers of the unit. These firing patterns have been interpretted in

terms of a model which assumes that the neurons driving a motor unit share a common excitatory input and are mutually linked by lateral inhibitory connections. The part of the nervous system generating this patterned firing has been called the flight oscillator (Wilson, 1966; Wyman, 1966, 1969a, 1969b) Levine (1973) has analysed the flight oscillator of Drosophila.

While recording intracellularly from the dorsal longitudinal muscles of shibire^{ST-139} it was observed that, as the temperature approached 30°C, the muscles began to fire spontaneously in a manner reminiscent of natural flight. Fig. illustrates the constancy of the interspike interval and the phase-locking of two of the muscle fibers in a motor unit even though the size of the action potential became greatly reduced as the temperature was raised. A pulse of cervical stimulation under these conditions produced a muscle action potential of the same reduced size. Temperature-induced firing of the flight oscillator could be maintained for many minutes in flies mounted in wax which did not beat their wings although the wings were free to move.

The above phenomenon did not occur in para, comatose or wild type flies. It appears that, in shibire^{ST-139}, elements that normally inhibit the flight oscillator are themselves inhibited at high temperature, so that the oscillator runs freely, providing input to the muscles. The latter respond with progressively lower action potentials as the temperature is raised. Incidentally, this response of the flight oscillator in shibire provides a convenient method for studying flight oscillations in solidly tethered flies unimpeded by the initiation of wing beats.

DISCUSSION

The nervous and the musclar systems of Drosophila are very complex and a given genetic lesion may affect various parts of the system to different degrees. Genetic alteration or loss of a specific membrane molecule, for instance, could, at one stroke. change the function of various neurons, synapses or muscles. As the system is wired up in a complex fashion, dysfunction of an inhibitory neuron might appear as an excitatory effect elsewhere. It is here that the possibility of constructing mosaic flies, with some parts mutant and the others normal, offers an incisive method for perturbing specific parts of the system in order to obtain information about its functional organisation.

In the para mutants the cessation of leg movements at high temperature is not due to the leg muscles themselves, since cervical stimulation at the paralysing temperature will still cause them to jerk. The flight muscles also remain excitable by direct intracellular stimulation. A clue to the site of the lesion is provided by the fact that, as the temperature is raised, the threshold for triggering the cervical nerve progressively increases. Even in this condition, once the signal reaches the muscle, a normal muscle action potential occurs. The para mutation thus appears to affect the excitability of the nerve.

The cervical connective contains over 3000 axons including a pair of giant fibers which attain a diameter of 10 µm (Hengstenberg,). These are likely to be the ones which are most readily stimulated and may be responsible for transmission of impulses to the legs and the flight muscles in these experiments. Electrophysiological and anatomical evidence

in favour of this assumption has been presented by Levine (1973). Perhaps the circuits which normally control spontaneous leg movement involve finer axons. These might be affected by mutation in the same way as the giant axons, but due to their smaller size, develop intractably high thresholds at a somewhat lower temperature. Thus, spontaneous leg movement would cease before cervically stimulated jerks, as the temperature is raised.

Alternative explanations are, however, possible. Suppose the effect of the para mutation is to block a certain class of synapses. Spontaneous leg movements, operating through one of these, will be blocked at the paralytic temperature. The fibers in the cervical connective might include some that control the legs via pathways involving synapses that are unaffected by the mutation. At elevated temperatures, a high stimulus voltage might trigger some of the alternative fibers which, perhaps due to their smaller size, are not triggered by lower stimuli.

The flight muscles of comatose remain excitable at the paralysing temperature, yet the action potential produced by cervical stimulation decreases in a graded manner. This graded decline of action potential resembles the effect of neuromuscular blocking agents on arthropod muscles. The fibrillar muscles of diptera are multiterminally innervated (Tiegs, 1955). The muscle membrane is electrically excitable, as in vertebrate twitch fibers, but the excited response is not an "all or none" propogated action potential. The entire muscle fires at once giving a graded response whose size is proportional to initial depolarisation (McCann and Boettiger, 1961; Usherwood, 1969). Neuromuscular blocking agents such as tryptamine or ether produce a graded diminution of the response (Hill and Usherwood, 1961; McCann and Reece, 1966).

Our observations on comatose suggest that raising the temperature progressively eliminates individual junctions in a multiterminally innervated muscle. This would be expected if the neuromuscular junction itself or the finer terminals of the axons are inactivated by temperature. When the temperature is sufficiently high, all the neural input becomes blocked and no muscle spikes are produced by cervical stimulation even though flight muscle is still directly excitable. Failure of nerve conduction is demonstrable directly in the larval nerve, though the properties of the nerves in the larvae could, of course, be different from the adults. In the ERG the receptor potential remains, but the positive spike fades out, which can be understood in terms of a failure of action potentials in the second order neurons.

When the temperature is raised with shibire^{ST-139} the electrical excitability of the muscle membrane is clearly impaired. The fact that flight oscillator continues to function even though the muscle spike becomes greatly reduced shows that the nerve cells and their axons continue to function. The temperature-sensitive lesion, so far as the flight muscle is concerned, must therefore be postsynaptic. The failure of the leg response to cervical stimulation may also be due to the leg muscle although this is not proven. But this is clearly not the whole story for shibire. The setting off of the flight oscillator suggests neural dysfunction somewhere; so does the failure of the positive spike of the ERG, a neural defect which mosaic evidence indicates to be postsynaptic. Conduction in the larval nerve, on the other hand is not blocked in this mutant.

We have examined the locomotor behaviour of mosaic flies carrying different paralytic mutations. The results of these experiments will be

described elsewhere. One particular aspect of the paralysis of temperature-sensitive mosaic flies may, however, be briefly noted here. In comatose as in para^{ts} and para^{ST-42} individual legs behave as independent units during paralysis and may or may not be paralysed. In the case of para^{ST-109} where paralysis requires a higher temperature, all six legs behave as a unit; in a given mosaic fly either all legs are paralysed together or none. Clearly in para^{ST-109} a more central element in the neural network controlling leg movements is affected by the paralysing temperature. Using mapping methods described by Hotta and Benzer (1972) the embryological focus of this centre can be easily located. The temperature-sensitive mutants thus provide a convenient method for selective mapping of nerve centres.

These experiments represent an exploration into the possibility of using mutants to produce perturbations in various physiological parameters of the nervous system and the muscles. Each of the three mutant types examined has revealed a different physiological syndrome. Although the small size of Drosophila would at first sight appear to be prohibitive, it turns out that it is possible to make several kinds of electrophysiological measurements with relative ease. It seems not unreasonable to hope that the great genetic advantages of this organism will be usefully applied to neurophysiology.

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DIVISION OF BIOLOGY

September 9, 1975

Dr. Obaid Siddiqi
Tata Institute of
Fundamental Research
Homi Bhabha Road
Bombay 400 005 India

Dear Obaid:

Enclosed is a copy of the Cold Spring Harbor manuscript, on which I have combined the various criticisms of the people in our group. Although some of them might be argued with, their negative reactions, if taken as indications of the reactions of other (and perhaps less friendly) individuals, are pretty devastating. It seems best to withdraw it for now. Rather, let's concentrate on getting fuller documentation and publishing elsewhere. *

I will try another version, in which the present data act as a framework for what needs to be filled in. In the meantime, get your oscilloscopes fixed, while I get mine into action, and let's both try to resolve the paradox and fill in the gaps. To do some of the experiments in duplicate might actually be beneficial.

One of the first things I plan to do is to repeat the Jan's experiments on larvae. One possibility is that the spike you were recording was not in the motor neuron. Another is that there is sufficient electronic spread to reach the endplate with a big signal. It would be desirable to record both the conducted action potential and the muscle response simultaneously in the same preparation. Also, using TTX should help clarify things.

i.e. in the jaws' opponent

I examined the behavior of stoned and it is rather unimpressive. Your description of depolarized sounds very different, and it seems unlikely that they are alleles, unless stoned is a very weak one. Nevertheless, if they are in the same chromosome region they ought to be tested for complementation.

We are embarking on a hunt for autosomal paralytics in the hope of obtaining additional sorts of mutants, since the yield of novelties on the X may be diminishing.

With best regards to Asiya and the kids,

Sincerely,

Seymour

SB:pe Seymour Benzer

* P.S. I just spoke to CSH. They will return the manuscript and figures to me. If we can come up with something within a month, it is still possible to get it in. She will check with me in a month's time.

– with collected criticisms and corrections

NEUROPHYSIOLOGICAL DEFECTS IN TEMPERATURE-SENSITIVE MUTANTS OF DROSOPHILA

Obaid Siddiqi
Molecular Biology Unit
Tata Institute of Fundamental Research
Bombay

and

Seymour Benzer
Biology Division
California Institute of Technology
Pasadena

I'd get . . .

Blackcomments by Byers – the writing is rotten. Introduction does not give the point of the paper. Discussion also no good – doesn't say what needs to be said. Should first elaborate all the possibilities (and give the answer for the mutants) at the beginning. Then give the documentation then repeat the answers at end. Lacks boldness.

Don comments:

As it stands, this is grist for Stent's mill. – It should not be submitted.

red = Bill's comments

black = Jan's comments

– Subject not to publish
– idea OK, but conclusions not clear
– expand the movie part
– could be rescued by using it as a general idea thing, with a few examples of preliminary results – still very flawed

Running Title: Neurophysiological mutants of *Drosophila*.

Send proofs to

O. Siddiqi
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1. INTRODUCTION

Neurophysiological experiments often require blocking of impulses across axons or synapses. This may be done by surgical interruption, or by local application of blocking agents such as chemical inhibitors or low temperature. Each of these methods has its own disadvantages. Neurosurgical lesions are irreversible, while blocking agents might be difficult to confine to a specified target, especially within the deep recesses of the central nervous system. A method of manipulating biological processes, widely used in molecular genetics, involves the use of temperature-sensitive mutations. Temperature-sensitive mutations result in the formation of an altered macromolecule, usually a protein whose configuration is thermolabile. The protein functions normally within a specified range of temperature but, at "non-permissive" temperatures, it undergoes a change which blocks the activities that depend critically upon its normal configuration. The mutant phenotype is thus expressed conditionally and can be evoked at will by changing temperature. The application of this methodology to the nervous system is an attractive possibility.

Our interest in temperature-sensitive mutants of Drosophila melanogaster was first aroused by the work of David Suzuki and his associates. Suzuki, Grigliatti and Williamson discovered a mutant of Drosophila, para^{ts}, which was instantly paralyzed at temperatures above 29^{o}C. When brought back to room temperature, the flies recovered in a few seconds. Subsequently, other temperature-sensitive paralyzed mutants were found (Suzuki et al., 1971; Siddiqi and Benzer, 1972; Grigliatti et al., 1973). The paralysis of these mutants could be due to temperature-dependent changes in components of the neuromotor system.

We have isolated a number of paralytic mutations on the X chromosome of D. melanogaster. Some of these are alleles of para^{ts} and shibire^{ts} (shi^{ts}) mutants previously described by Suzuki and his associates. Others belong to a new gene comatose (com). Recently, Satpal Singh, R.N. Singh and Sheela Donde analysed a crop of mutants which includes, besides alleles of the previously known genes, a new mutant tentatively called depolarized. Electrophysiological tests show that these mutants carry distinctive temperature-dependent lesions in their nerves, muscles or synapses.

It is possible, by genetic techniques, to construct mosaic flies in which only a part of the body is mutant. The expression of the paralytic genes in such mosaics is circumscribed to the mutant portions of the fly (Suzuki et al., 1971). A combination of mosaic technology with mutations affecting elements of the neural circuits provides an incisive method of analyzing the neurophysiological mechanisms of behaviour (Hotta and Benzer, 1972). We describe here some physiological properties of paralytic mutants of Drosophila. Experiments with mosaics show that paralyzed mutants can be used to map nerve centres that control motor behaviour.

2. PARALYTIC MUTANTS

Figure 1 shows the locations on the X chromosome of various temperature-sensitive paralytic mutations discussed here. Several alleles of the genes $para^{ts}$ and shi^{ts} have been described by Suzuki and his collaborators (Suzuki et al., 1971; Grigliatti et al., 1973). The map shows new alleles of these, as well as comatose and depolarized, isolated at Caltech and Bombay. The mutant depolarized has not yet been tested for complementation against stoned (Grigliatti et al., 1973).

The mutants of each gene have characteristic responses to elevated temperature, the paralyzing temperatures varying widely among alleles as among the different genes. The most sensitive alleles of para and shi become paralyzed at 27° C while others may require up to 37°C. The mutant comatose is paralyzed by a short exposure to 38°C and depolarized passes out at 33°C. The temperature at which paralysis occurs for some of the mutants depends upon the duration of exposure to high temperature. For instance, comatose can be paralyzed at temperatures as low as 30°C if exposed long enough.

The paralytic mutants also differ from each other in recovery at room temperature. The recovery of $para^{ts}$ and its alleles is very quick, taking only a few seconds. On the other hand, comatose recovers very slowly, after a lag which depends upon the extent of exposure (Figures 2 and 3). The mutants shibire and depolarized recover quickly after a short paralysis but may take a long time after extensive exposure at high temperatures. In all the mutants examined so far, adult flies as well as larvae are affected although the paralysis of larvae might require a different temperature.

3. ELECTROPHYSIOLOGICAL CORRELATES OF PARALYSIS

A few easily performed tests enable one to detect whether impulses in sensory or motor pathways are blocked during paralysis. An electric shock of 2 to 3 volts to the cervical nerve of the fly elicits a jerk in the legs. The response can be monitored either visually or through an electromyogram (EMG) recorded extracellularly from the tibia. The wild type moves its legs spontaneously and also responds unfailingly to shocks at temperatures up to 42°C. In para^{ts} and its alleles, spontaneous leg movements cease at the paralyzing temperature, but the cervically stimulated response continues. Thus, while some of the pathways in para are blocked, others remain operative. In comatose, when the fly is taken to 38°, at which temperature it becomes rapidly paralyzed, both spontaneous movement and stimulated response become blocked; the same happens to shi^{ST139} when it is exposed to 32°C. The mutants shibire and comatose, therefore, appear to have lesions between the excitation of the cervical nerve and the contraction of the muscles in the leg.

The electroretinogram (ERG) of Drosophila, evoked by a short flash of light, contains two prominent components, a corneal positive on-transient and a corneal negative wave (Hotta and Benzer, 1969). The negative wave reflects depolarization of the photoreceptor cells, triggering the on-transient which apparently arises from the second order neurons of the lamina. The ERGs of normal flies and para remain essentially unchanged up to 38°C except for a shortening of latency. In both comatose and shi^{ST139}, the negative wave remains unaffected, but the positive spike is reversibly eliminated by high temperature (Siddiqi and Benzer, 1972). A similar result using long-flash ERG has been

obtained by Kelly and Suzuki (1974) who find that, in shi~~ts~~, high temperature blocks the on and off transients without affecting the receptor potential.

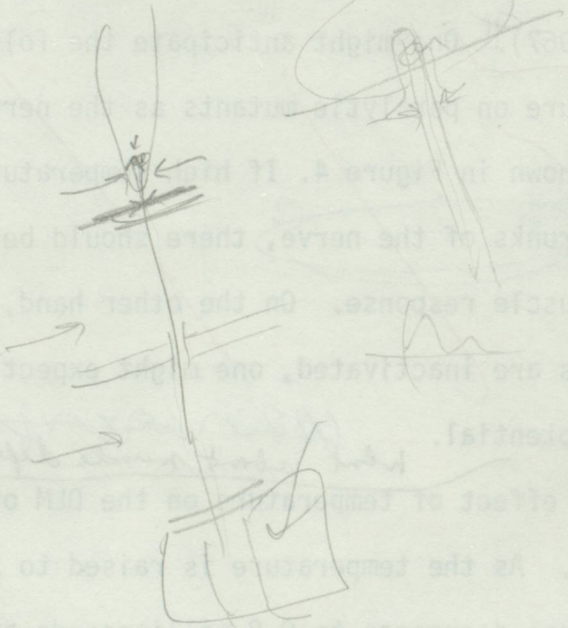
The EMG and ERG are ~~mass~~ responses from many cells. More definitive information about the nature of the block in the mutants can be obtained by recording from single cells. The flight muscles provide a convenient preparation for this purpose.

Intracellular recording from flight muscles

The dorsal longitudinal muscles (DLMs) are large fibrillar muscles, six on each side, that indirectly control wing beat by contracting and relaxing the thorax. Each muscle is a single cell, the largest being about ~~120 by 60~~ μm in cross section, and is easily impaled with glass microelectrodes. The DLMs are innervated by motoneurons in the thoracic ganglion and by the two giant axons of the cervical connective, ~~which is a bundle of 3500 fibres.~~ Each giant axon is about 10 μm in diameter. Anatomical and physiological evidence indicates that collaterals of each giant axon terminate directly on the DLMs of the contralateral side. Other fibers of the cervical conn. are connected to DLMs through one or more interneurons in the thorax (Power, 1948; Tiegs, 1955; Coggshal et al., 1973; Levine and Hughes, 1973; Levine and Tracey, 1973; Levine, 1974). The recording preparation and a schematic circuit of the pathway are shown in Figure 4. The flies were fixed with dental wax, and 3 M KCl-filled glass microelectrodes were introduced through an opening in the cuticle. The resting potential of the muscles ranged from 70 to 85 millivolts and cervical stimulation elicited a single action potential with a small overshoot (McCann and Boettiger, 1961). The delay from the stimulus to the beginning of the action potential was 1.2 milliseconds, consistent

with the assumption that the fibres most readily stimulated in these experiments are the giant axons that terminate directly upon the DLMs. The fibrillar muscles of Drosophila are multiterminally innervated (Tiegs, 1955). The muscle membrane is electrically excitable like that of the twitch fiber vertebrate, but the action potential is probably not propagated. The electrically excited component of the response is dependent on the strength of the depolarizing stimulus. Due to multiple innervations and a long space constant, the entire fiber fires at once, and the recorded action potential is the sum of a number of local responses (McCann and Boettiger, 1961; Usherwood, 1969). Neuromuscular blocking agents such as ether, 5-hydroxytryptamine or tryptamine produce a graded diminution of the response (Hill and Usherwood, 1961; McCann and Reece, 1967). One might anticipate the following possible effects of temperature on paralytic mutants as the nerve is stimulated in the manner shown in Figure 4. If high temperature blocks the action potentials in the trunks of the nerve, there should be a sudden, "all or none" loss of the muscle response. On the other hand, if nerve endings or neuromuscular junctions are inactivated, one might expect a progressive decline of the muscle action potential.

The effect of temperature on the DLM of a normal fly is shown in Figure 5. As the temperature is raised to 39°C, the lag from stimulus to response decreases to 0.8 milliseconds and the time scale of the response contracts. The resting potential and the amplitude of the action potential are not affected. Thus, the pathway from cervical nerve to DLM remains essentially intact.



But, When the temperature of para^{ts}, para^{ST42} or para^{ST109} is raised, there occurs a sudden "~~all or none~~" failure of the action potential at a certain critical temperature. If the temperature is reduced below this critical value, the action potential reappears (Fig. 6). The temperature at which the response fails is somewhat variable from fly to fly and is usually a few degrees above the temperature required for locomotor paralysis of the strain. In a given preparation, the responses of all DLMs fail at the same temperature. At the point where the response has just failed, it can be evoked again by raising the stimulus amplitude, but the threshold rises very rapidly and, within a minute or so, the preparation becomes inexcitable by a ten times stronger stimulus. Upon recovery, the threshold comes down to the usual 3 volts. In many preparations, a small signal can be seen preceding the action potential by half a millisecond (Fig. 6). This is, most likely, the presynaptic nerve impulse. When the action potential fails, this signal also disappears. The "all-or-none" failure of the action potential, the increased threshold of excitation, and the extinction of the presynaptic signal all tend to show that high temperature blocks the excitation of the ~~nerve~~.

The effect of temperature on comatose is of a different kind. When the fly is raised to 38°C, the response undergoes a series of changes. The amplitude of the spike is gradually reduced and a prominent inflection in the rising phase appears. The action potential falls gradually to what looks like an end-plate potential. Finally, the junction potential itself vanishes. Within about a minute at 38°C no trace of neural input into muscle can be seen (Fig. 7). The recovery at room temperature is also graded and follows the same sequence in reverse. The graded decline

and recovery of the action potential in comatose, in contrast to the "all or none" effect in para suggests that temperature causes failure of individual nerve endings or synapses or both.

The behaviour of shi^{ST139} is similar to that of comatose except that, ~~in this case,~~ the graded decline in action potential begins at a lower temperature (30°C) and the muscle action potential is gradually reduced to a small end-plate-like potential (Figure 8). On decreasing temperature, the action potential rapidly recovers its normal size and shape. The reduced response at high temperature may persist for many minutes, but if the temperature is raised above 32°, the response becomes greatly attenuated and recovery takes several minutes. The chief difference between shi^{ST139} and comatose seems to be that in shi^{ST139}, although the muscle response is drastically affected, the nerves continue to fire at the non-permissive temperature. Evidence is presented in the following section to show that temperature does not block the propagation of nerve impulses in shibire but affects the neuromuscular junction.

In the mutants described above, temperature does not affect the resting potential significantly. In contrast, the resting potential of DLMs in the mutant depolarized rises towards zero above 34°C; on reversing temperature, the cell re-polarizes. A detailed account of this mutant will be given elsewhere.

Excitability of muscle membrane:

In order to test whether temperature affects the excitability of the muscle itself, the response of DLMs to intracellular current injection

was examined. A longitudinal flight muscle was impaled with two glass microelectrodes. ~~Through one of these a pulse of~~ depolarizing current was injected ~~while~~ the other ~~electrode recorded the response~~. The response to cervical stimulation was monitored simultaneously. The result typical of normal flies is shown in Figure 9. A 30-millisecond pulse of 200-600 nanoamperes induced a repetitive discharge of spike-like potentials. In many preparations the direct response persisted unchanged on repeated stimulation, while in others, continued current injection reduced the excitability of the muscle. The majority remained excitable up to 39°C. At high temperatures, the response often changed from sharp spikes to a nearly sinusoidal discharge. In some of the preparations the spikes became attenuated and were eventually lost irreversibly, presumably due to damage by the large currents injected.

The mutants comatose and para^{ts} behave essentially in the same manner as the normal fly. At a temperature where the indirect response to cervical stimulation was blocked, the response to direct stimulation clearly persisted (Figs. 10 and 11). The effect of temperature on para and comatose, therefore, cannot be due to inexcitability of the muscle membrane.

In shi^{ST139}, under the conditions used by us, the response to direct stimulation tended to fail at temperatures above 32°. Electrical excitability of the muscle recovered on return to room temperature, but not fully, ~~perhaps because of cell damage~~. Ikeda, Ozawa and Hagiwara (personal communication) have examined the effect of temperature on the flight muscles of shi^{ts}. They observed that, at high temperature, the input resistance of flight muscles decreased, in both mutant and normal

flies but on injection of sufficient current, the muscles of shibire were still excitable above its paralyzing temperature.

Propagation of impulse in larval nerve:

In order to examine the effect of temperature on the propagation of impulses in nerve axons, it is necessary to record from a pathway in which no synapses intervene between the site of stimulation and the site of recording. In the larva of Drosophila, eight pairs of nerves run from the ventral ganglion to the abdominal body segments (Hertweck, 1931). The exposed nerves can be stimulated at one end and the ensuing action potentials recorded at the other with a suction electrode. Since, in the mutant strains examined, larvae as well as adult flies are paralyzed by high temperature, the larval nerves can be used to test the effect of temperature on impulse conduction.

The nerves of the normal larva continued to fire at 39°C (Figure 12). On the other hand action potentials in para^{ts} were blocked between 32° and 35° and in comatose at 38° (Figure 13). ~~These results are consistent with the experiments on flight muscles which indicate neural lesions in para and comatose.~~ The mutant shi^{ST139} was indistinguishable from normal in that impulse propagation survived even at 39°C, ~~which is~~ well above the temperature for paralysis ~~of both larvae and adults.~~

4. TEMPERATURE-INDUCED FLIGHT FIRING

The motor neurons innervating the fibrillar flight muscles of dipterans fire in a regular clockwork fashion. The interspike intervals are constant and the fibers terminating on the muscles of the same motor unit are phase-locked, that is to say their respective spikes tend to occur in a stable temporal order. The characteristics of neural input into flight muscles are explained by a model which assumes that the neurons driving a motor unit share a common excitatory input and are mutually linked by lateral inhibitory connections. The ensemble of cells generating the patterned firing of flight muscles constitutes the neural flight oscillator (Wilson, 1966; Wyman, 1966 and 1969). The flight oscillator of Drosophila has been analysed by Levine (1973).

As the temperature of shi^{ST139} approaches 30°C, the DLMs begin to fire in the manner characteristic of flight. While the muscle action potentials become reduced in size, as described earlier, patterned firing continues for many minutes (Fig. 14). This firing of the neural oscillator was not accompanied by wing beats although the wings were free to move.

It would seem that, in shi^{ST139}, elements that normally inhibit the flight oscillator are themselves inhibited at high temperature so that the oscillator runs freely. Patterned generation of reduced junction potentials at high temperature provides independent evidence that the propagation of nerve impulses is not blocked and that the defect lies at the junction. Incidentally, such temperature-induced flight firing provides a convenient method for studying the neural control of flight in solidly tethered flies, and Wong and Ikeda (personal communication) have made use of this. Kelly and Suzuki (1974), while recording the ERG

of shi^{ts}, observed an oscillatory discharge at elevated temperature which was presumably generated by flight muscles. Induced flight firing did not occur in normal flies, nor in para or comatose, which is consistent with the finding that, in both these mutants, nerve impulses are blocked. On the other hand, depolarized exhibits temperature-induced firing somewhat similar to shi^{ST139}.

5. MOSAIC MAPPING OF NERVE CENTRES

Gynandromorphs of Droposphila arising from a fertilized egg heterozygous for sex-linked recessive mutations are genetic mosaics, the male portion exhibiting the mutant phenotype. Imagine a mosaic fly carrying a temperature-sensitive paralytic mutation affecting some component of the nervous system. A part of the system can now be switched off by simply raising the temperature. This is closely akin to making neurosurgical lesions with the advantage that the structure as a whole remains intact and the lesions are reversible. The mosaic mapping technique (Sturtevant, 1929; Garcia-Bellido and Merriam, 1969) produces a formal representation of the fly on a two-dimensional surface, each point on the map representing the blastoderm anlage of some future structure of the organism. Hotta and Benzer (1972) have shown that mosaic mapping can be used to identify the embryological origins of behavioural traits.

Suzuki, Grigliatti and Williamson (1971) found that, in flies mosaic for para^{ts}, male portions of the fly were paralyzed while the female portions (heterozygous for para^{ts}) remained active. We have examined the leg movements of several hundred mosaics of para^{ts}, para^{ST42} and para^{ST109} as well as comatose. In mosaics of para^{ts} and para^{ST42}, most legs with mutant cuticle were paralysed while most legs with normal

You don't say anything about Hk foci – refer to prior?

cuticular markers remained active at high temperature. But a certain proportion of legs exhibited "recombinant" behaviour, that is to say externally normal legs were paralyzed and mutant legs were active. Any leg could be paralyzed independently of the others. We could, therefore, determine a separate focus on the fate map for each leg. These foci, shown in Fig. 15, are located in a region of the fate map which corresponds to the thoracic ganglion. Similar results were obtained with comatose.

What is the significance of this vis a vis what has been said earlier

how shown?

as shown in the fig 15, this is like saying a similar focus was found for legs I & II

The result with para^{ST109} was strikingly different. In a given mosaic fly at 36°C, either all legs were paralyzed or all legs were active. The focus for paralysis of the legs was calculated according to Hotta and Benzer (1972), using the domineering focus model. This focus maps to a region lying between the areas of the map corresponding to the cuticles of head and thorax. Evidently, the paralysis of para^{ST109} involves a more central defect in the control of leg movement than does the paralysis of para^{ts} or comatose. What structure is derived from this area is not known. Ghysen (personal communication) has mapped several "sluggish" mutants, defective in locomotor activity to this region.

mention shi here.

People are going to want to know where shi's fate maps. If defect is at the junction, as you indicate, the obvious question is: is it pre- or post synaptic in the junction? They expect fate maps to shed light on this!

– mention shi here
– Kelly eye
– Hall interval mutes
– Kankel ST134

– we need a discussion of possible interpretation for different alleles of para mapping to different foci.

6. CONCLUSIONS

The mutants described here represent examples of ~~possible~~ genetic perturbations of the nervous system. As temperature is raised, paralysis must reflect dysfunction of that part of the system which fails first. At high temperature, ~~at least~~ some of the nerves of para become inexcitable, while the neuromuscular junction is ~~normal.~~ The nerves of shibire continue to fire, but the junctions are blocked. In comatose, impulse propagation along the nerve trunks ~~and their finer endings close to synapses may be affected.~~ Failure at the endings precedes the blocking of the main axon. ~~The mutant~~ depolarized loses resting potential in its ~~muscles and perhaps also in its nerves.~~

~~No doubt, many more such mutations will be found.~~ From a closer study of the mutants, one might hope to learn something about neurophysiological mechanisms. At present we know little of the structural causes of paralyzing lesions. Kelly (1974) reported that shi^{ts} exhibits an increased tolerance to tetrodotoxin, which, in mosaics, is confined to the mutant tissue. This has led him to suggest that the regenerative sodium channel is altered. Our experiments on DLM and larval nerve show that nerve conduction in shi^{ST130} is unimpaired, so that the block is most probably synaptic. Other mutants might be defective in potassium, calcium or chloride gates; such mutations could provide additional means of probing the organization of ionic channels. Yet other mutants might be affected in the synthesis, release or degradation of neurotransmitters, or in receptor proteins.

A single gene mutation can produce diverse effects. The alteration or loss of a specific membrane molecule, for instance, could, at one stroke, change the function of various nerves, synapses or muscles. ~~As~~ Since

the system is wired up in a complex fashion, dysfunction of an inhibitory neuron might appear as an excitatory effect elsewhere. Using mosaics, normal and mutant parts may be put together in various combinations, and the defects can be switched on or off by temperature. This makes it possible, in principle, to map the nerve centers that control complex behaviour.

to control action

we know there are crummy, shrinking cells, but...

ACKNOWLEDGEMENTS

The research done at Caltech was supported by the National Science Foundation and by a Gosney Research Fellowship to O.S. The work at Tata Institute was supported by the Jane Coffin Childs Memorial Fund.

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Figure legends are inadequate. Give enough detail so that figure or legend are self-sufficient. Explain the arrows. Define all traces. State what temperature etc used.

LEGENDS TO FIGURES

Fig. 1. Locations of temperature-sensitive paralytic mutations on the X chromosome of D. melanogaster. A number of alleles of the mutants paralyzed^{ts} and shibire^{ts} have been isolated in Suzuki's laboratory. Mutants with superscripts ST were isolated at Caltech. Mutants with superscripts TP were isolated at the Tata Institute.

Fig. 2. Paralysis and recovery of para^{ts} in response to temperature shifts. Different batches a) b) c) d) were shifted to 38° at T=0 and then to 23°C at t = 1, 2, 5, 10 min respectively.

Fig. 3. Recovery of comatose at 23°C after exposure to 38°C for various times.

Fig. 4. a. Setup for recording intracellular responses of longitudinal flight muscles.

b. Schematic diagram of neural connections from the cervical connective to a dorsal longitudinal muscle. One pathway is direct; the other involves one or more intervening synapses.

Fig. 5. Effect of temperature on the indirect DLM response of a normal fly stimulated at the cervical connective. } confusing

Fig. 6. Effect of temperature on the indirect DLM response in para^{ST42}. Above a critical temperature, there is an abrupt failure of response. The response reappears as the temperature is lowered. During the transition, the muscle is prone to fire more than once (see also Fig. 7).

Fig. 7. Effect of high temperature on the indirect DLM response in comatose. The amplitude declines and the latency increases. During recovery, the sequence is reversed. Two successive sweeps are shown in each figure.

Fig. 8. Effect of temperature on the indirect DLM response in shi^{ST139}.
1 - 4: temperature increasing from 28°C to 32°C
5 - 10: temperature decreasing to 25°C.

Fig. 9. Response of DLM in a normal fly to direct stimulation by injected current (Right). Indirect response to cervical stimulation in the same preparations is also shown.

Fig. 10. Response of DLM in para^{ST42}. The indirect response failed at 37°C but the direct response was maintained.

Fig. 11. Response of DLM in comatose at different temperatures. Indirect and direct stimuli are given in the same sweep. At high temperature, the indirect response vanished but the direct response remained.

Fig. 12. Propagation of impulse in larval nerve of normal Drosophila. The impulse persists as temperature is raised to 39°C. The mutant shi^{ST139} gave similar results. Recording artifact.

Fig. 13. Effect of temperature on propagation of impulse in larval nerve of comatose. In the mutant para^{ST42}, the nerve impulse is also blocked.

Fig. 14. Temperature-induced flight-firing in shi^{ST139}.

a. Flight oscillations begin at 28°C. Patterned firing is maintained as temperature is increased to 32°C, even though the amplitude of the muscle response is greatly reduced.

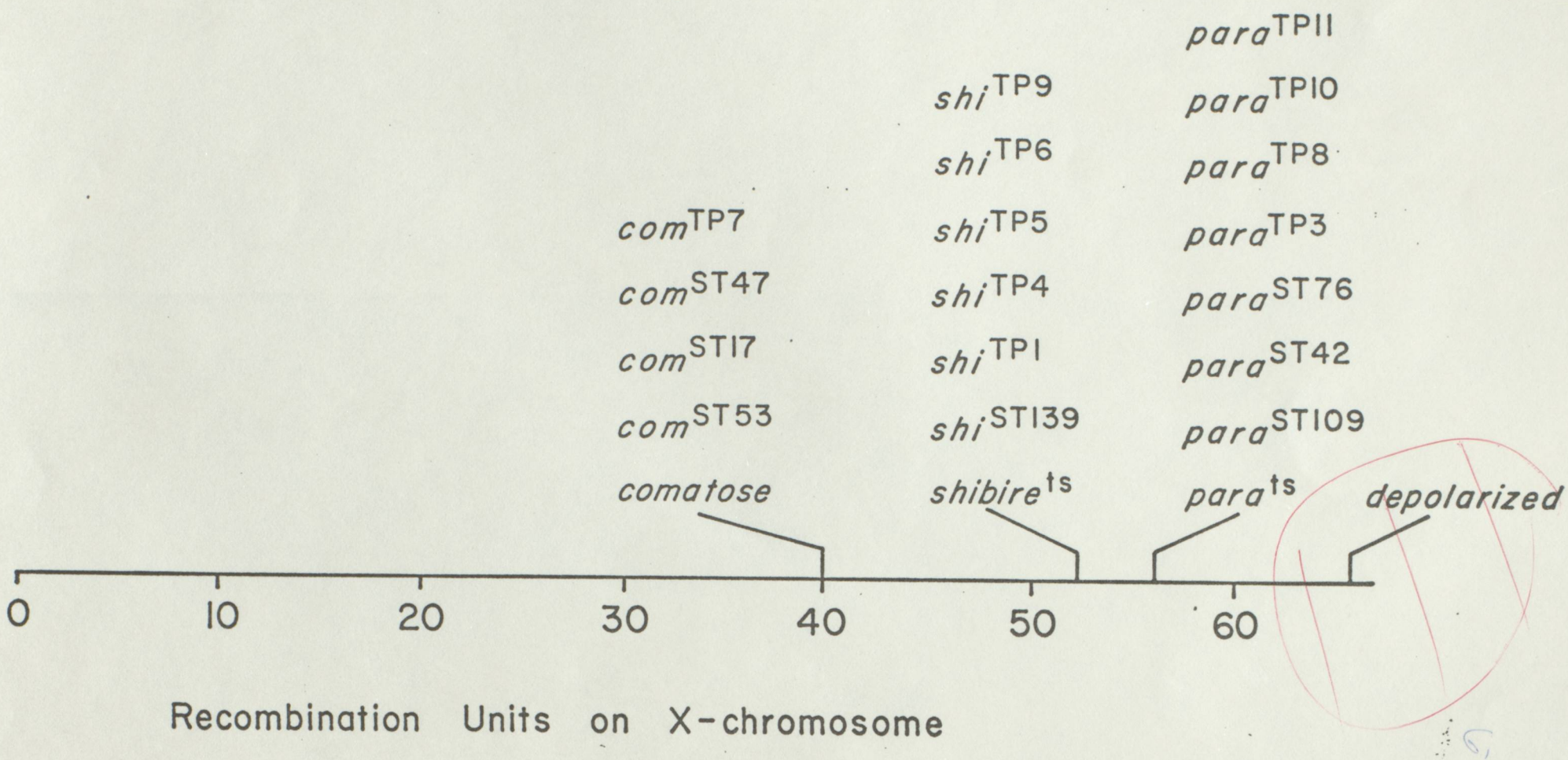
b. Simultaneous recordings from two DLM fibers on the same side. Temperature 33°C. Multiple sweeps were triggered by the spikes of cell #1. Though the spikes were greatly reduced, patterned firing was maintained.

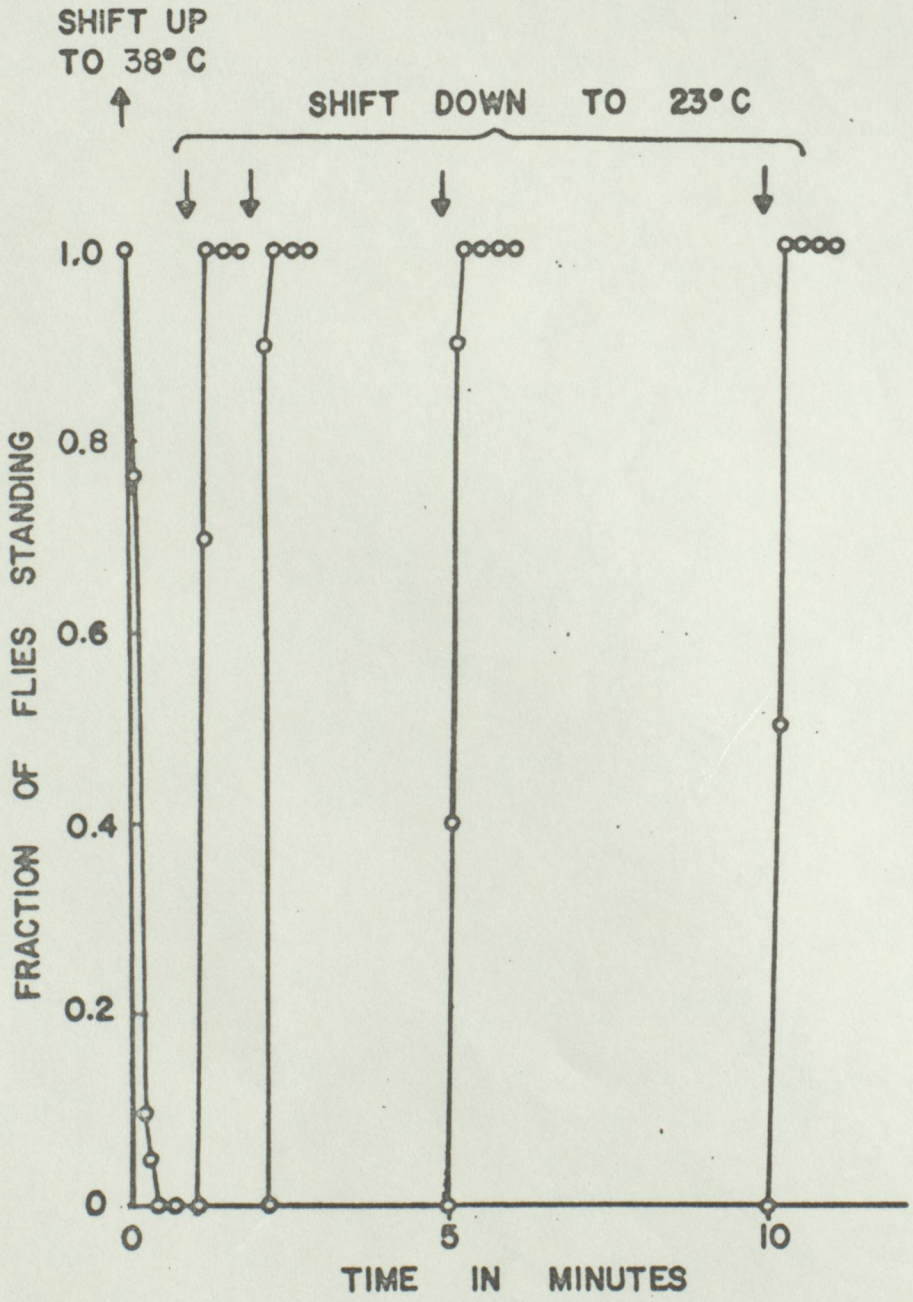
Fig. 15. Fate maps of foci of paralytic mutants obtained by scoring leg movement in mosaic flies above the paralytic temperature. Distances are in sturts (Hotta and Benzer, 1972).

a. *para*^{ST42}. There is an independent focus for each leg, in the region corresponding to the thoracic ganglion.

b. *comatose* gives a similar result.

c. *para*^{ST109}. The legs are paralyzed as a group. The focus maps to an entirely different region.


com^TP7
com^ST47
com^ST17
com^ST53
comatose
shi^TP9
shi^TP6
shi^TP5
shi^TP4
shi^TP1
shi^ST139
shibire^ts
para^TP11
para^TP10
para^TP8
para^TP3
para^ST76
para^ST42
para^ST109
para^ts
depolarized
0
10
20
30
40
50
60
Recombination Units on X-chromosome


SHIFT UP
TO 38° C
SHIFT DOWN TO 23° C
1.0
0.8
0.6
0.4
0.2
0
FRACTION OF FLIES STANDING
0
5
10
TIME IN MINUTES

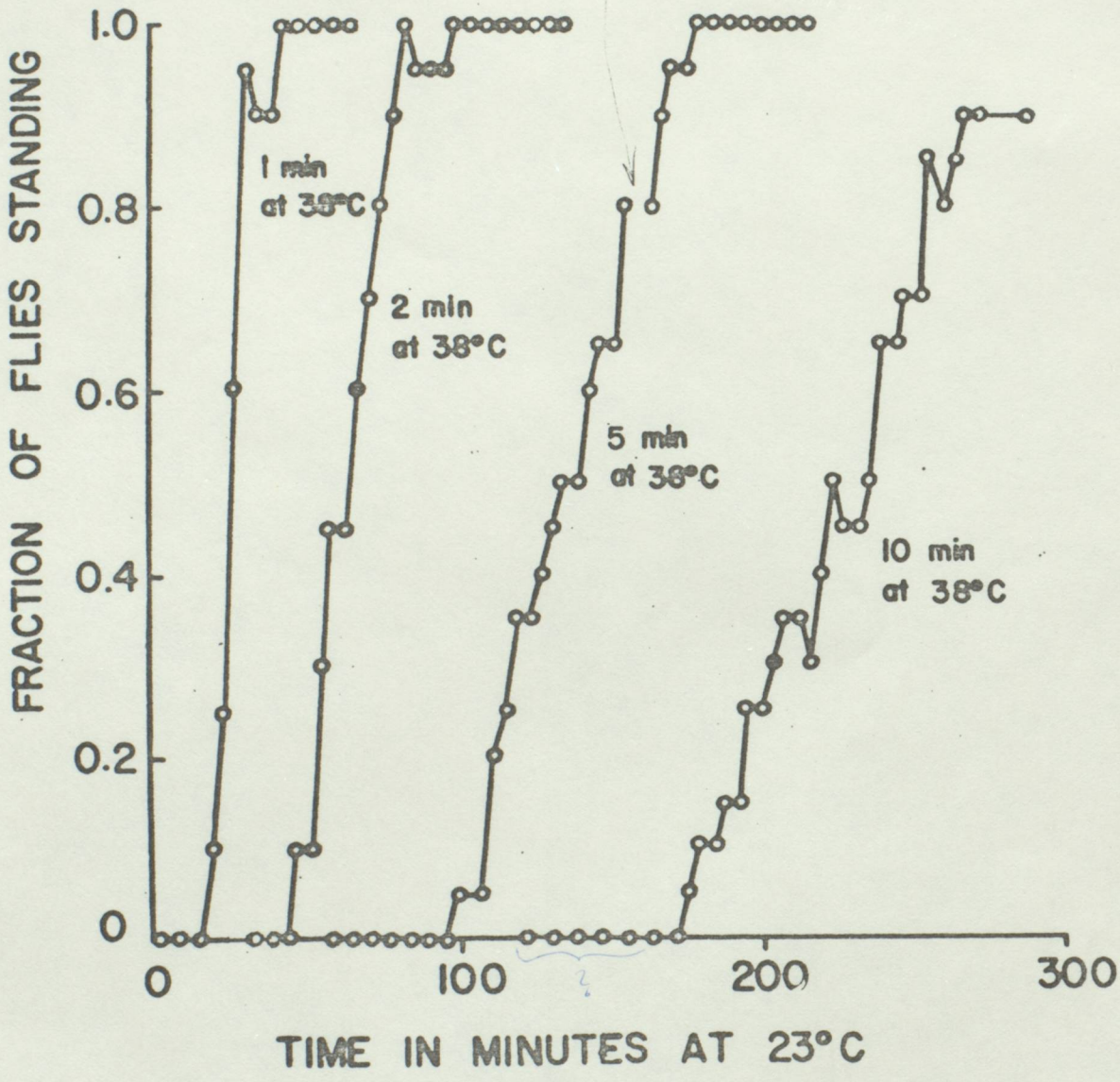
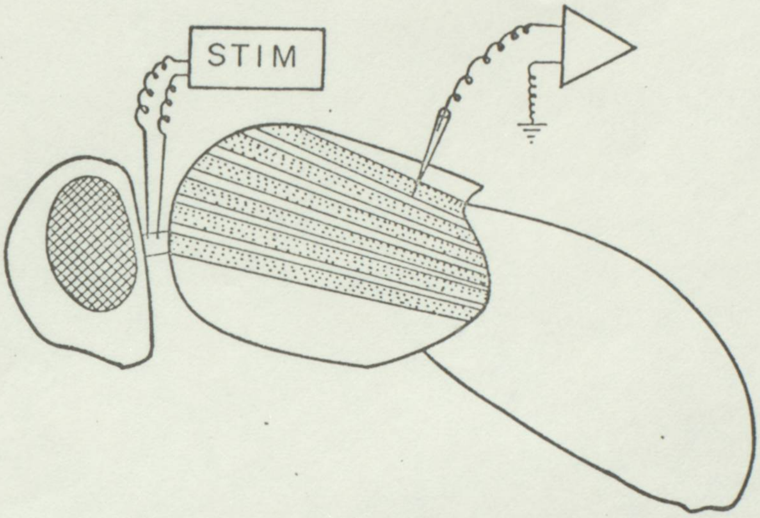
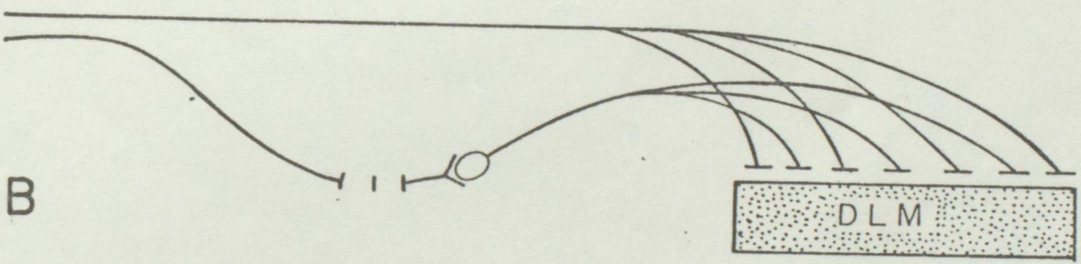

FRACTION OF FLIES STANDING
1.0
0.8
0.6
0.4
0.2
0
1 min at 38°C
2 min at 38°C
5 min at 38°C
10 min at 38°C
0
100
200
300
TIME IN MINUTES AT 23°C

Fig 3

A



B



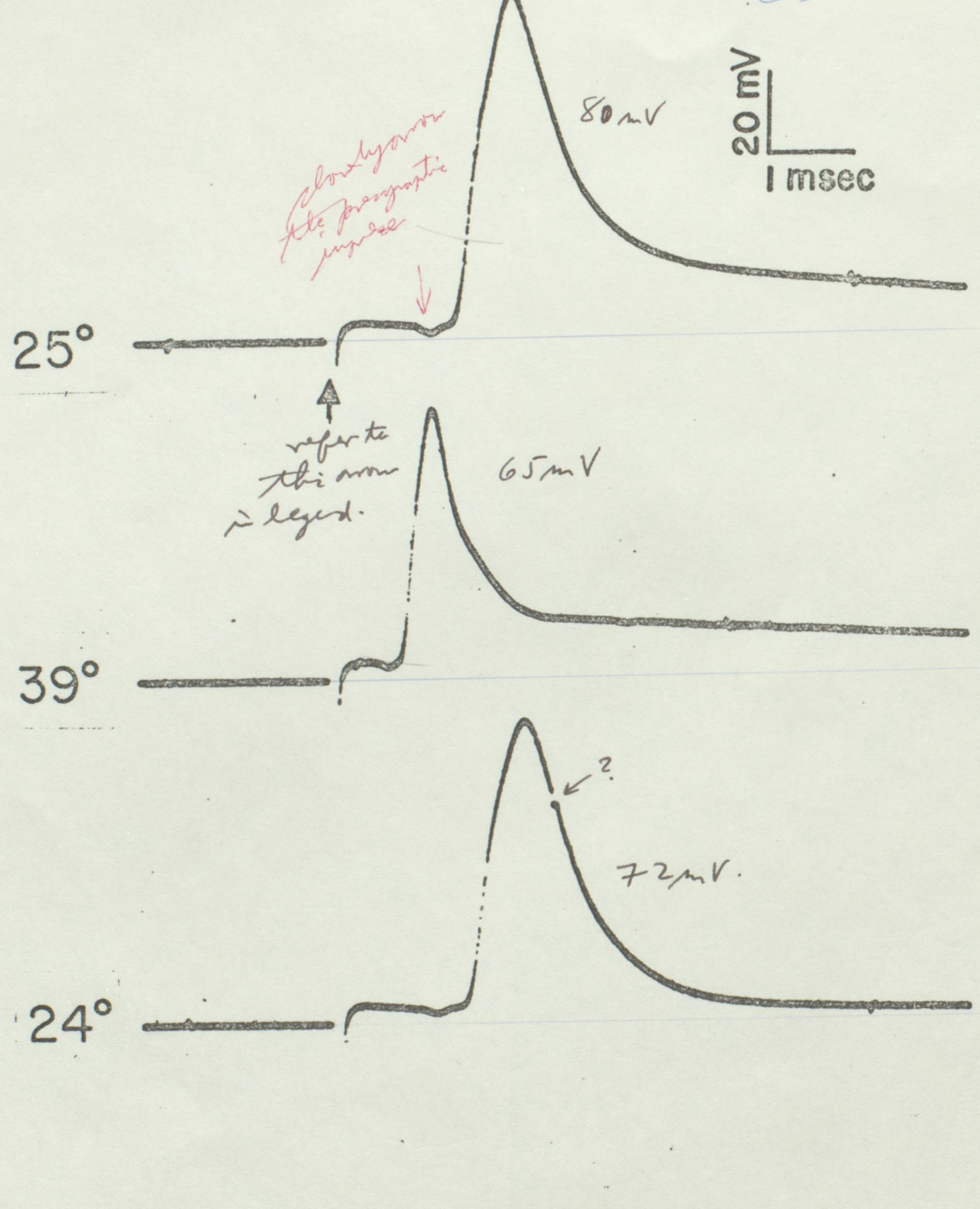
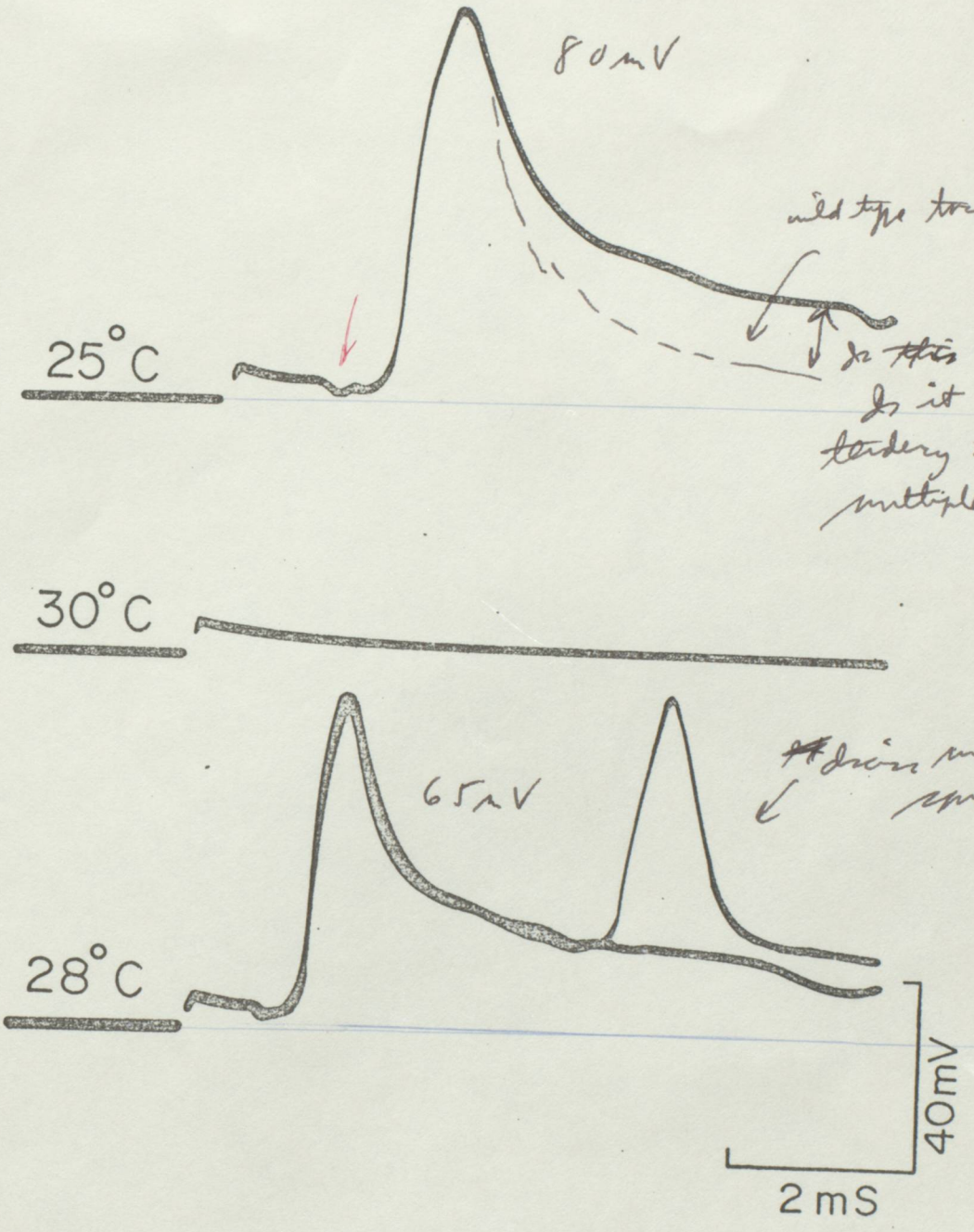


Fig 5 - correct [illegible]

[illegible]

paralyzed ST42



related?

mild type traces from Fig 5

Is this difference significant? Is it related to tendency of axon to fire multiple spikes?

drives multiple spikes.

Same scale as Fig 5. (Could omit this scale marker)

italics

ST42

yeh - my theory ①: it's a release defect: impulse invades nerve ending, but Ca gate opens very slowly, so release does not occur until later.

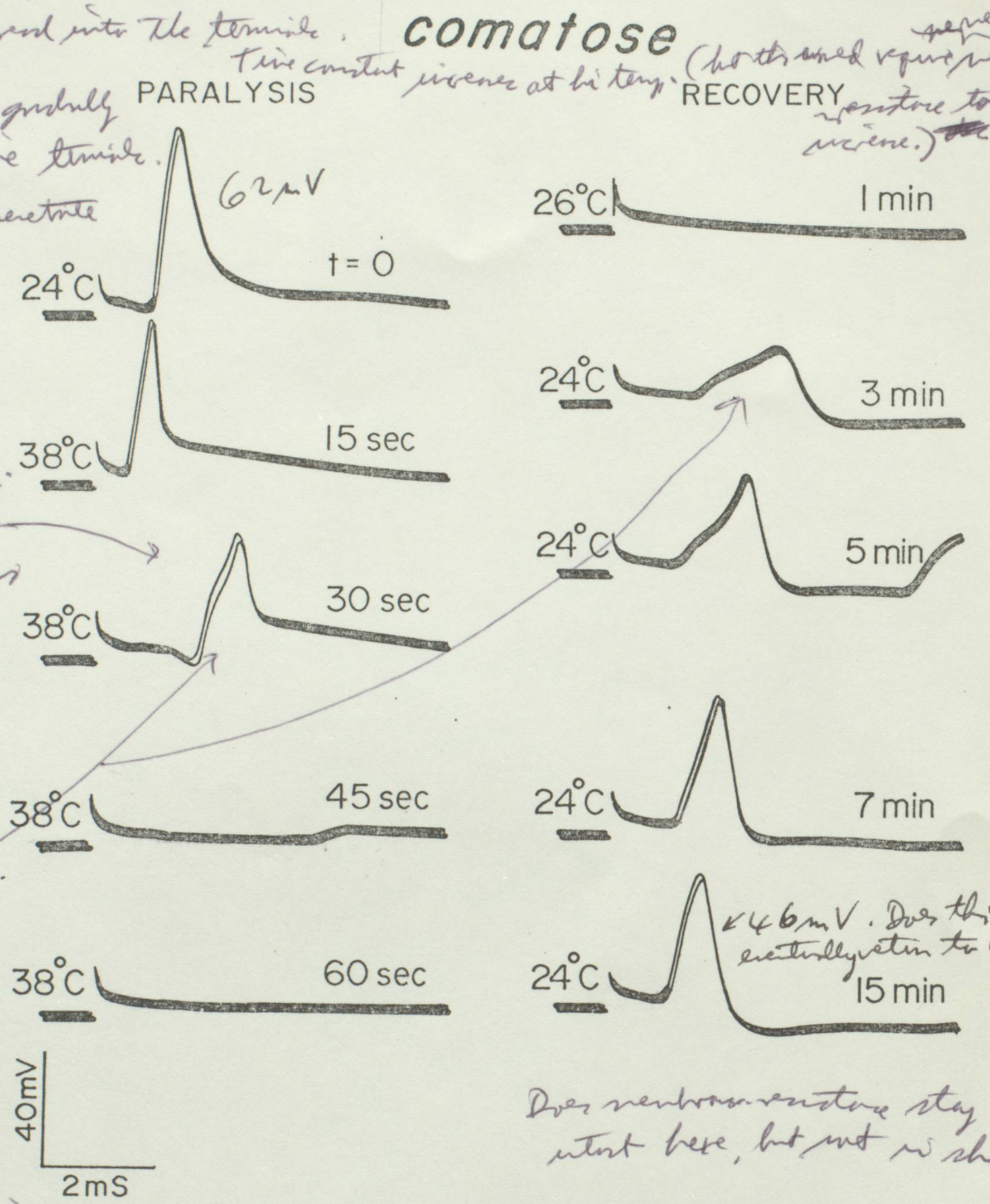
theory ② passive spread into the terminals. Time constant increases at hi temp. (how this would require membrane resistance to increase.)

Suppose nerve conduction gradually blocked, first at fine terminals. + passive spread must penetrate over ~~to~~ progressively longer lengths of terminal as T increases. This gives longer latency + also smaller amplitude.

could one measure this directly?

state that the shift is graded, not abrupt (i.e. it is not another pathway)

is this response graded?



62 mV

← 46 mV. Does this eventually return to 62 mV?

Does membrane resistance stay intact here, but not in shi?

Shift from conducted spike to passive spreading? How does spreading latency depend on temp?

I know, the spike goes faster at hi temps (even though reduced in amplitude)

Fig 7 Time constant for passive spread should change as membrane resistance decreases. In what direction?? (Jess dunno) Adelman p. 52 says time const. is proportional to resistance

~~[illegible]~~

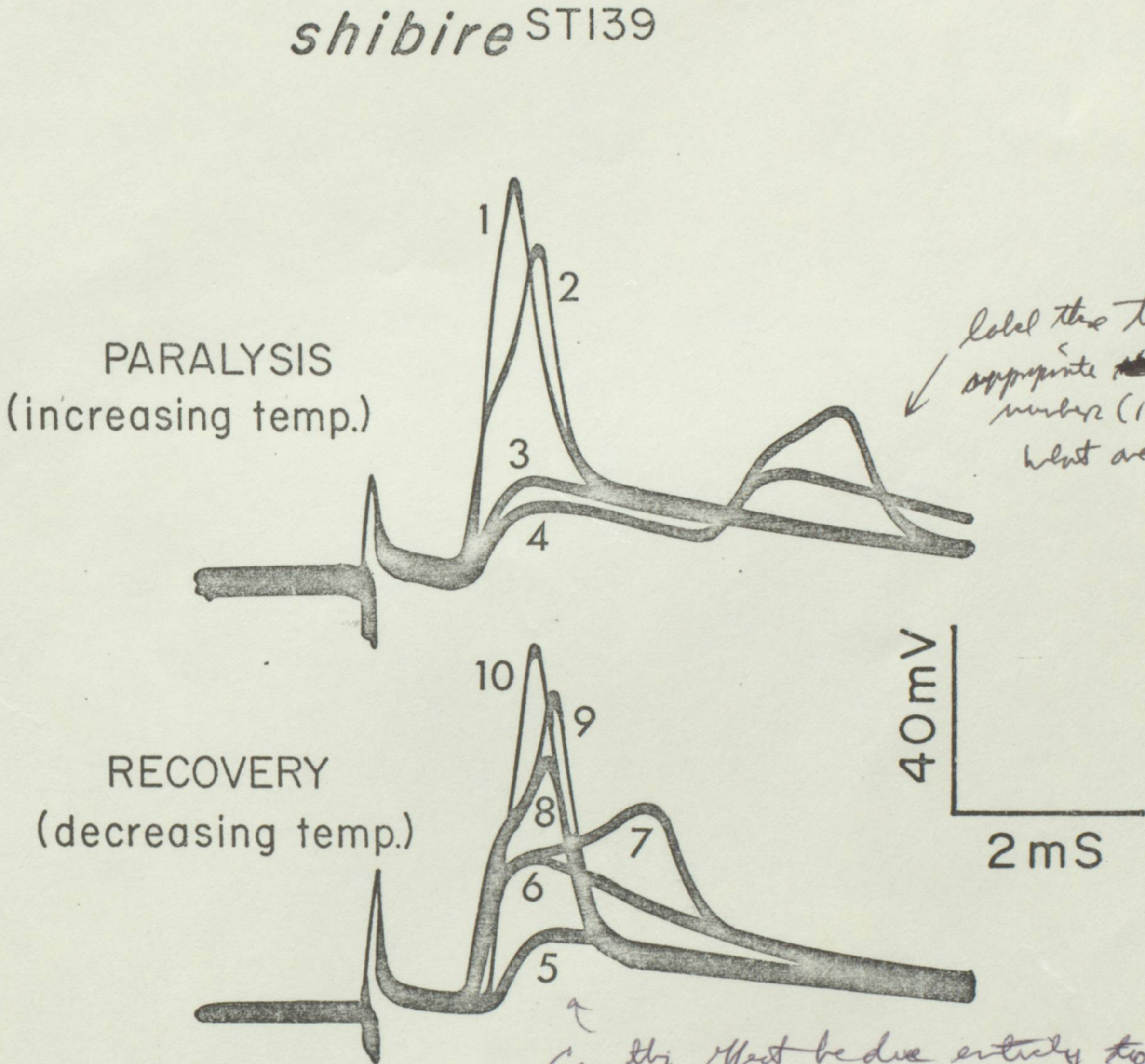
~~change comatose to comatose (stoned)~~

prob. not applicable

Jones: have never seen formulation for delay in propagation of passive spread

the latency in shi does not change!

How does resistance of membrane normally change c̄ temp?

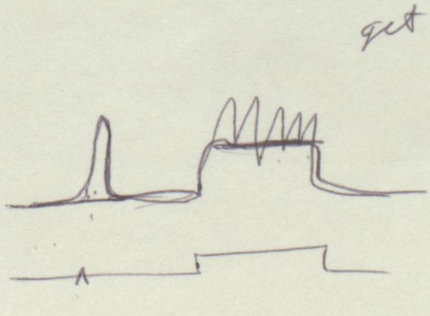


label these two with appropriate ~~[illegible]~~ number (1→4). What are they actually?

Can this effect be due entirely to decrease in membrane resistance?

would also explain lamina

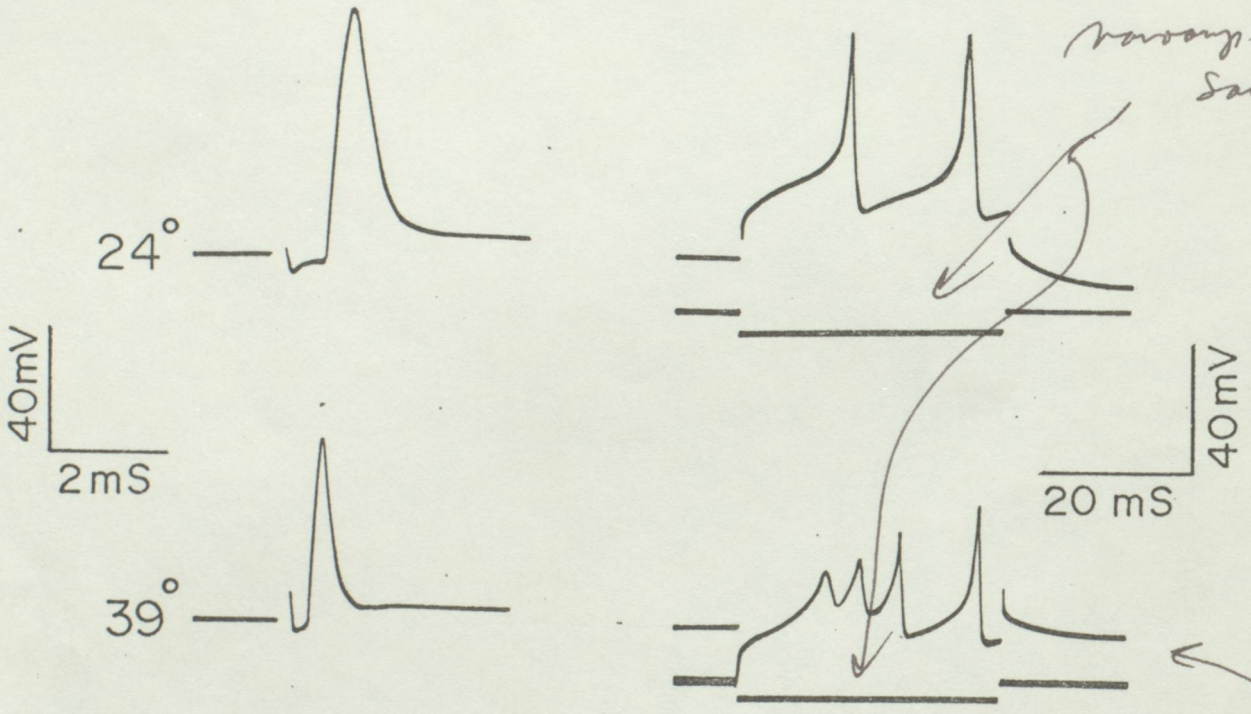
If you think transmission fails, because he can still get response by injecting current.



Put time of each trace in the figure legend.

Fig 7

normal fly

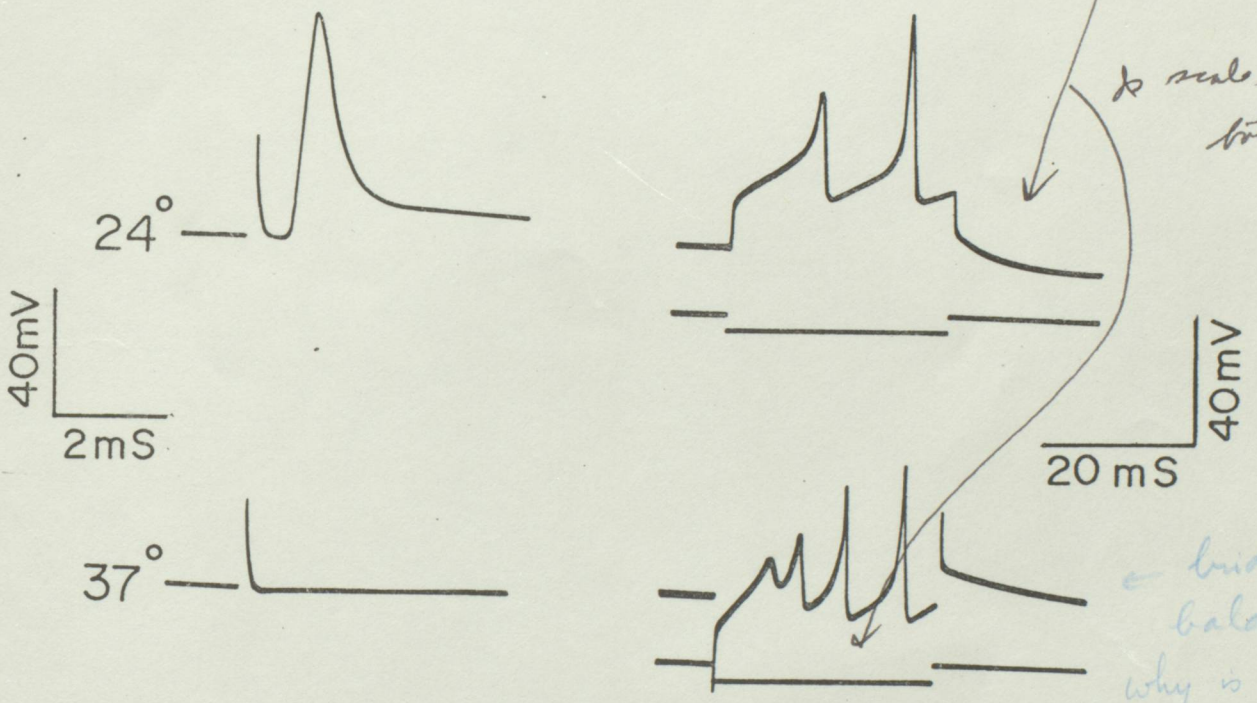


CERVICAL STIMULATION

DIRECT STIMULATION

what is the voltage scale?

paralyzed ST42



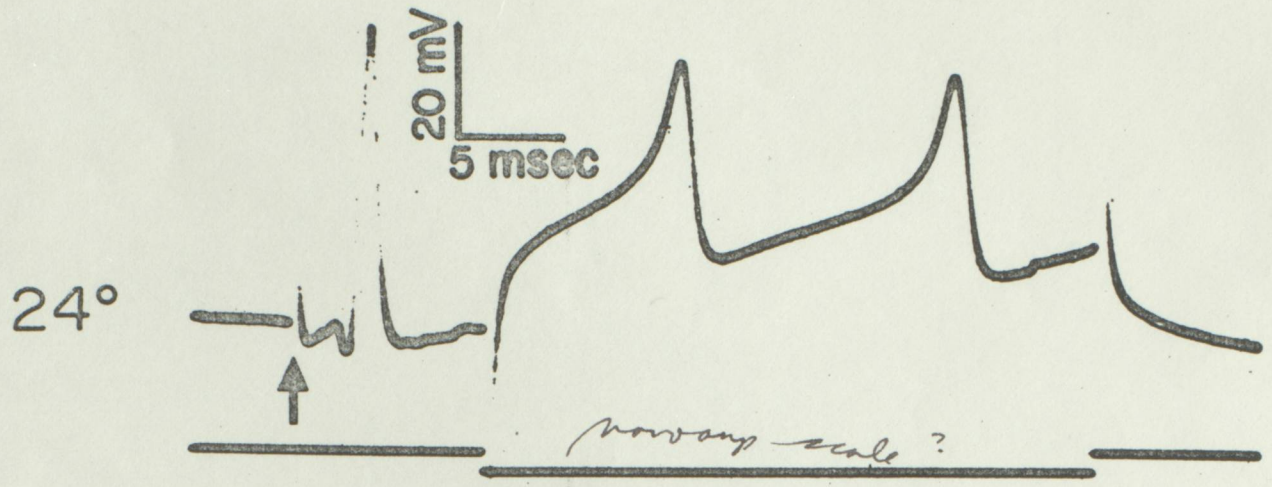
do scale same in both?

CERVICAL STIMULATION

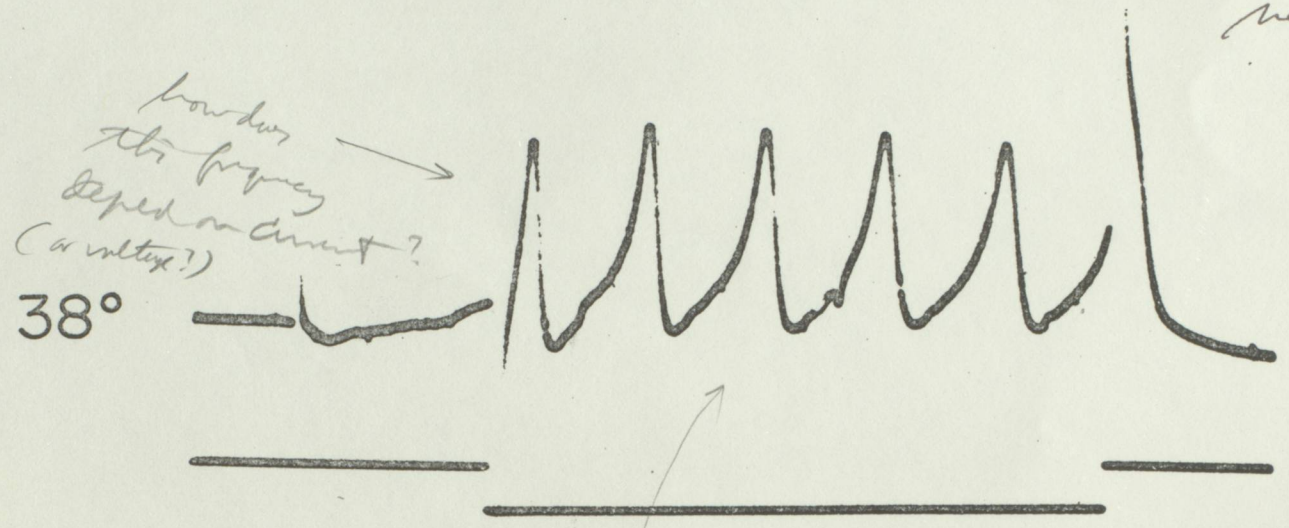
DIRECT STIMULATION

bridge not balanced? why is there an apparent hyperpolarization when stimulated

comatose



bridge balance constant? what is change in membrane resistance?

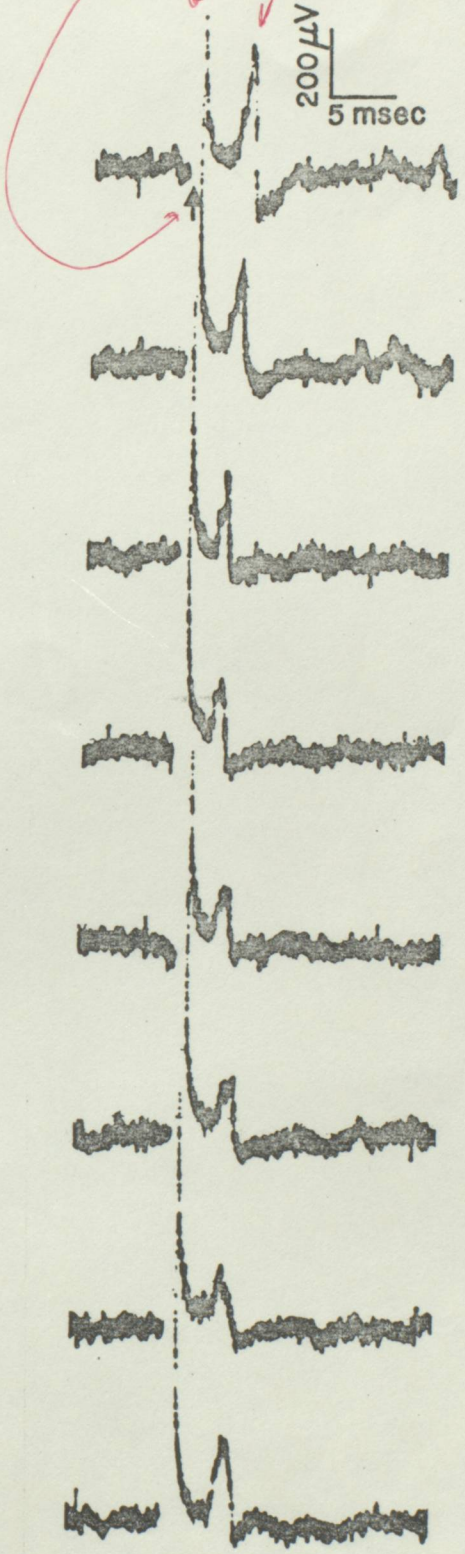


narrow slow frequency dependent current? (a voltage?)

higher freq due to higher membrane resistance?

normal larva

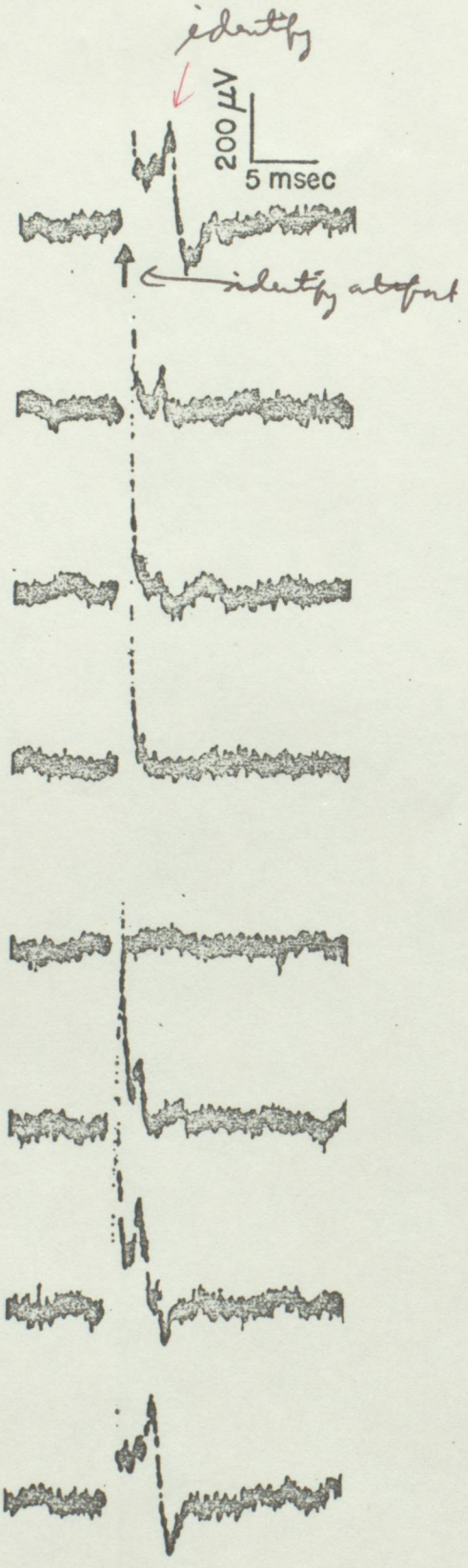
time	temp.
0 min	26°
0.5	36°
1.0	39°
2.5	40°
3.0	37°
3.5	30°
4.0	28°
5.5	27°



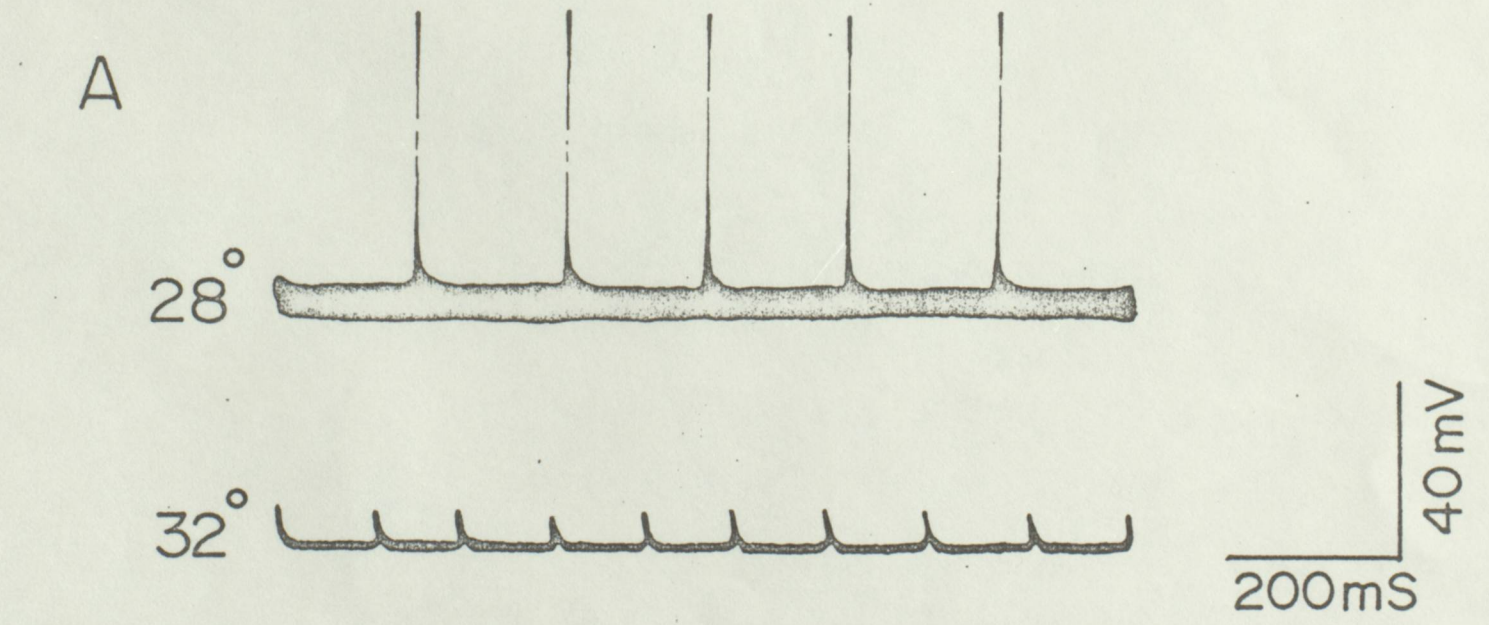
What is being recorded here?
Is it really an action potential?
Does it retain some sign after stimulus pulse is passed?
Does it show a threshold?
What is effect of TTX.
Possible spatial summation by different length ⇒ amplitude
Constant but time lag changes?
It's not clear that signal is due to motor axon.
The jaws muscle exerts motor axons while this is activity of another axon.

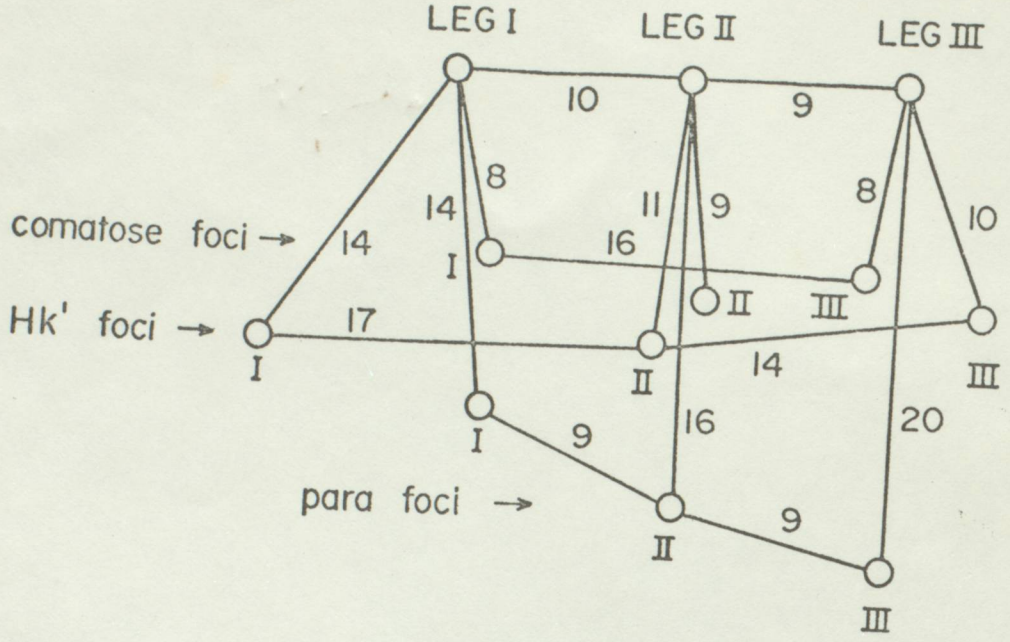
comatose larva

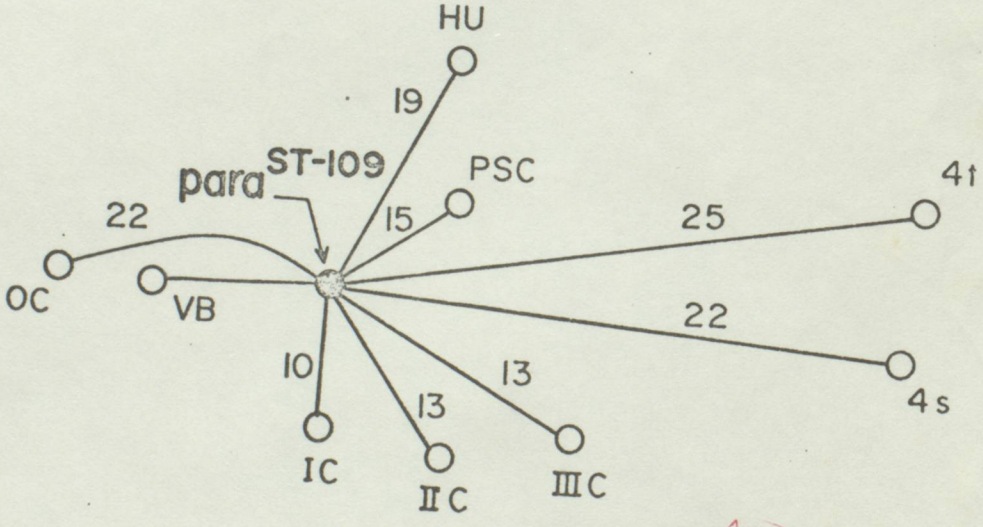
time	temp.
0 min	26°
0.5	37°
0.8	38°
1.0	39°
3.0	34°
4.0	32°
5.0	29°
8.0	26°



shibire^{ST139}







data for this?

redraw rays for each mutant

redraw rays completely, showing full half-tetrads and more carefully

Neurophysiological Defects in *comatose* and other Temperature-Sensitive Paralytic Mutants of *Drosophila melanogaster*

INTRODUCTION

Suzuki, Grigliatti and Williamson (1971) described a mutant gene *paralysed*, on the X-chromosome of *Drosophila* that causes instant paralysis at temperatures above 30°C. When the temperature is reduced the fly recovers quickly. Another mutant *shibire*^{ts} behaves similarly (Grigliatti et.al., 1973). Paralytic mutations could affect different parts of the nervous system. In certain mutants, paralysis might be due to a failure of axonal conduction; in others transmission at a particular kind of synapse might be blocked; yet others might be defective in the neuromuscular junction or the muscles. These defects could, for instance, emanate from mutations in genes responsible for the synthesis of membrane components or transmitter molecules. Mutations of this kind would provide a potentially powerful tool for analysing the neuromotor system since the defect can be turned on and off at will and the same animal serves as its own control. In addition one can use genetic techniques to construct mosaic flies in which some parts of the animal are mutant and others normal, thus localising the focus of the lesion.

We have isolated several temperature-sensitive mutants and investigated the physiological basis of paralysis in normal, mutant and mosaic flies. Some of our isolates are alleles of *para* and *shibire*, others belong to a new temperature-sensitive paralytic gene. The new

gene comatose, is located at 40 units on the X-chromosome. The comatose flies become paralysed after a brief exposure to 38°C. Whereas the recovery of para^{ts} and shi^{ts} from the same treatment occurs in a matter of seconds, recovery of comatose requires minutes or hours, depending upon the length of exposure to high temperature. In normal flies paralysis does not set in until 42°C and recovery is quick.

Neurophysiological studies reveal distinctions between different paralytic mutants. When the flies are fixed on their backs in wax, the legs show persistent spontaneous movements at room temperature. An electric shock to the cervical chord evokes a sharp jerk in the legs. At the paralysing temperature of 30°C, para stops spontaneous leg movements but when cervical stimulation is applied, the legs still respond.

The result of a similar experiment with the mutant comatose is different. As the fly is raised to 38°C, spontaneous leg movement ceases, but so do the leg jerks in response to cervical stimulation. This defect persists on return to room temperature until the period required for recovery from paralysis is over; comatose appears to develop, in the paralysed state, a block somewhere between the cervical connective and the muscles. The experiments described here were done to localise and identify this block.

The mutant shi^{ST-139}, isolated by us, also stops leg movements, and loses the leg response to cervical stimulation above its temperature of paralysis. Electrophysiological analysis, however, shows its properties to be different from comatose. Thus, mutations in all three temperature-sensitive paralytic genes affect the neurological mechanisms in distinct ways.

MATERILAS AND METHODS

1. Isolation of mutants

Males of the strain Canton-Special (CS) of Drosophila melanogaster were mutagenised with ethyl methyl sulfonate and mated to attach-X females marked with yellow and forked according to the procedure described by Lewis and Bacher (1966). Each F_1 male was again mated to a virgin attached-X female to produce a set of F_2 males carrying identical mutagenised X-chromosomes. Adults of each such line were exposed to 38°C to detect temperature-induced changes in behaviour. Among — lines screened, mutants were obtained that became sluggish or paralysed at the elevated temperature. Seven of the mutants are described in this report.

2. Genetic mapping and complementation tests

Female heterozygotes were constructed for all combinations of the seven mutants and tested by exposure to 40°C for one minute. This exposure was sufficient to induce paralysis in all the mutants whether hemizygous males or homozygous females but not in normal CS flies, nor in any of the mutant/+ heterozygotes. By this test the seven mutants could be separated into three complementation groups.

Through the courtesy of Dr. David Suzuki, samples of $para^{ts-1}$ and shi^{ts-2} were obtained and tested against our mutants, revealing that two of our groups coincided with his. The third group comatose, which requires a higher temperature for paralysis, represents a new gene. This gene was mapped by recombination against the markers — and — on the X-chromosome at position 40 ±.

3. Heating chamber for neurophysiological experiments

The fly was mounted in dental wax on a 0.5 cm cork fixed at the centre of a perspex block (Fig.) surrounded by a metallic shield made of tin can, 3 cm x 4 cm x $\frac{3}{4}$ cm in size and covered at the top with a bakelite lid. The lid had a perspex window for illumination and observation and openings on both sides for placing electrodes. The insulated shield was wrapped with a coil of tungsten wire. The current through the coil was supplied by a Versa-Therm proportional temperature device controlled by a thermister close to the fly. The temperature was measured by a second thermister (YSI No. 427). About 3 minutes were required to raise the heating chamber from room temperature to 38°C. When it was necessary to attain the paralysing temperature quickly, the chamber was prewarmed to the required temperature before inserting the mounted fly.

Subsequently the heating chamber was replaced by a simpler apparatus consisting of a bakelite sheet with a cylindrical cavity of dia. 2 cm and height 0.5 cm, mounted on a glass slide. The floor of the cavity was lined with a thermofoil heating element (Minco Products. Inc. Minneapolis). The fly was mounted in the centre of the cavity and the heating current through the thermofoil was regulated by a variable resistor. The temperature was monitored by a thermocouple microprobe (Cole Parmer No. 8506-30) placed under the abdomen of the fly. The heat capacity of this system was small so that the temperature could be raised or lowered within a minute or so.

4. Cervical stimulation for leg jerk

The fly was mounted ventral side up and the tips of the wings, the abdomen, and the front legs were waxed down. The head was fixed in

wax so as to slightly stretch the neck. Electrolytically sharpened stainless steel needles were used as stimulating electrodes. These were inserted on both sides of the cervical nerve and 2-3 volt pulses of 0.4 millisecond duration were delivered. The normal fly responded to each pulse with a strong jerk of legs.

5. Electromyograms

The response of the leg muscles could also be monitored electrically. The fly was mounted on its back and the legs on one side were stretched and waxed down at the tip. A micropipette filled with 1 molar NaCl was inserted in the tibia of the mesothoraccic leg. A second micropipette placed in the abdomen served as the reference electrode.

6. Intracellular recording from flight muscles

The dorsal longitudinal flight muscles of Drosophila can be easily impaled with glass microelectrodes filled with 3M KCl using the stereotaxic map of the muscle fibers constructed by Levine and Hughes (1973). The fly was mounted, dorsal side up, and its legs were stretched out and fixed. One side of the thorax was supported on a wax stilt taking care that the thoraccic spiracles remained open. The cuticular terminations of the individual muscle fibers can be located with the help of morphological landmarks on the surface of the thorax (Levine and Hughes, 1973). One or more holes were made in the cuticle with a sharpened needle through which the tip of the microelectrode could be inserted in a desired direction. In most of the preparations we could record from each of the six dorsal longitudinal fibers on one side, one after another. The identity of the fiber could, therefore, be established with sufficient confidence. The

reference electrode, a micropipette filled with NaCl, was placed in the thorax. The resting potential of the longitudinal muscles was typically around -70 to -80 mV and the action potentials observed in response to a 2-3 volt stimulus in the cervical chord were similar to those described by McCann and Boettiger (1961) for dipterans.

7. Direct intracellular stimulation of flight muscle

For direct excitation of flight muscles two separate micropipettes filled with 3M KCl, one for current injection and the other for recording, were inserted in the same fiber. A 30 millisecond pulse of 200-600 nanoamperes through the stimulating pipette was usually sufficient in normal flies to elicit a response.

8. Electroretinogram

The ERG in response to a 20 μsec strobe flash was recorded as described by Hotta and Benzer (1969). The flash was introduced into the heating chamber with a fiber light guide.

RESULTS

Some of the mutants isolated by us are allelic to $para^{ts}$ and shi^{ts}, mutants described by Suzuki and his associates (Suzuki et.al., 1971; Grigliatti et.al., 1973); others belong to a new gene comatose. The location of the paralytic mutants on the genetic map of the X-chromosome, their allelic relationship and phenotypic characteristics are summarised in table I.

The new gene comatose maps at 40 ± units. The three com alleles are paralysed at 38°C but differ in the duration of paralysis

at room temperature after a given exposure to high temperature. The kinetics of recovery of the adult com flies are shown in Fig. —— . The mutations are recessive and com/+ heterozygotes are not paralysed. in complementation tests, all combinations of the three mutants alles gave the mutant genotype. Out of these com^{ST-53} was selected for detailed study.

The three new alleles of the previously-described gene para^{ts} have properties qualitatively similar but differ in the temperature required for paralysis. All are recessive and do not complement each other or para^{ts}. Our mutant shi^{ST-139} is an allele of shi^{ts} (Grigliatti et.al., 1973) and has similar properties.

In all of the above mutants locomotor paralysis found in adult flies is also observed in larvae, although a somewhat higher temperature may be required. Thus larvae of para^{ts} become immobilised at 32°C and recover rapidly when the temperature is lowered. shi^{ST-139} behaves the same although recovery is slower after prolonged paralysis. The larvae of comatose like the adults, are immobilised by 38°C or more and remain paralysed for a long time at room temperature. The fact that the larvae show behaviour similar to the adult is useful as the larval nerves offer a convenient preparation to study the conduction of action potentials.

Neurophysiology

We have carried out a number of electrophysiological experiments on normal and mutant flies to determine whether any part of the nervous

or muscular system is affected when a mutant becomes paralysed.

1. Response to cervical stimulation: An easily performed test is to apply a mild electric shock to the cervical nerve and observe the response of the legs. Wild type flies responded to a 0.2 millisecond stimulus of 2 to 4 volts with a strong jerk of all the legs. As the temperature was raised the response persisted upto 40°C for several minutes.

The mutant comatose after one or two minutes at 38°C, did not show leg jerks in response to cervical stimulation even after return to room temperature. A ten-fold increase in stimulus to as high as 20 volts failed to cause any perceptible leg movement. After the requisite recovery period, however, the jerk response returned to normal. Flies exposed to 38°C for one minute recovered fully in about 5 minutes; a 5 minute exposure required more than half an hour for recovery.

The mutant shi^{ST-139} was affected in the same way as comatose, but at a lower temperature. The leg response stopped in about 2 minutes at 32°C, recovering within a minute or two, after return to room temperature. Longer exposures at 32°C - 34°C required longer periods for recovery.

Thus, in both comatose and shi^{ST-139} one or more of the steps along the pathway from cervical nerve to leg muscle are blocked.

In contrast, the para mutants $para^{ts}$, ST42 and ST109 continued to exhibit cervically stimulated leg jerk well above their paralysis temperatures. The paralysis shown by these mutants cannot be due to a generalised failure of nerve conduction, neuromuscular junction or muscle excitability. Something must be blocked, since the flies legs cease spontaneous movement at high temperature, but the experiment either overrides or bypasses that block.

2. Intracellular recordings from flight muscles

To test whether impulses from the cervical nerve actually arrive at muscles, a convenient station to record from is the set of very large indirect flight muscles in the thorax. Each of these is a single cell and can be readily impaled with a glass microelectrode. Intracellular recordings were made from the dorsal longitudinal muscles using micropipettes filled with 3M KCl. The resting potential in the normal flies ranged from -70 to -80 millivolt, and cervical stimulation evoked a single action potential with a small overshoot (McCann and Boettiger, 1961). An example is shown in Fig . The delay from the stimulus to the beginning of the action potential was 1.2 milliseconds. At 39°C the response time decreased to about 0.7 milliseconds and the spike became sharper but the resting potential and the amplitude of the action potential were undiminished for several minutes. Thus, in wild type flies, the entire pathway from cervical connective to flight muscle action potential remains intact at high temperature.

A different result was obtained for the para group of mutants $para^{ts}$, $para^{ST-42}$ and $para^{ST-109}$. When these were taken above 32°C keeping the stimulus pulse constant at about 3 volts, the muscle action potential suddenly dropped to zero at a certain critical temperature. When the temperature was lowered slightly, the action potential reappeared. This was an all or none effect that could be repeated many times by shifting the temperature above or below the critical point. All the longitudinal muscle fibers in a given fly ceased to fire at the same temperature; in different preparations, however the critical temperature varied. The histograms in Fig — show the variability in the three mutant strains. The effect is usually discernible only at temperatures well above the

temperature which causes locomotor paralysis. *para*$^{ST-109}$ flies require a higher temperature for paralysis than *para*^{ts} and *para*$^{ST-42}$.

At the point where the muscle response in *para* mutants first fails, it can be evoked again by raising the stimulus by a few volts. But the stimulus threshold rises very rapidly with time and the preparation becomes inexcitable by stimuli exceeding 10 volts. During recovery the earliest response is evoked by a large shock exceeding 10 volts but the threshold quickly comes down to the usual 3 volts. In some preparations a small signal can be seen which precedes the muscle action potential by about 0.5 milliseconds. This may be the presynaptic nerve spike; it disappears, or becomes greatly diminished, *pari passu* with the muscle action potential (Fig). The all or none failure of the muscle response and the increase in the threshold of excitation indicate that in these mutants, temperature affects the excitability of the nerve rather than muscle.

The effect of temperature on the flight muscle response of *comatose* was of a different kind. At 38°C the action potential underwent a reduction in amplitude and an increase in latency. The action potential first changed to a form that looked like a junction potential; that is to say it lost its inflection, and finally, in about 30 seconds disappeared altogether. Recovery at room temperature was slow and graded, showing a similar progression in reverse (Fig.). At the height of paralysis the presynaptic nerve spike" could not be seen; all traces of neural input into muscle had disappeared. The graded effect in *comatose* is distinct from the all or none loss of action potential in the *para* mutants.

The behaviour of *shibire*$^{ST-139}$ resembles that of *comatose* in

showing a graded effect. At 30°C the muscle action potential was gradually reduced to a small end-plate-like potential. At room temperature the action potential rapidly recovered its normal size and shape (Fig.). shibire^{ST-139} is affected at a much lower temperature than comatose and recovers faster. Also the reduced action potential may persist for a long period at 32°C. If the temperature is raised to 34°C or more, the response becomes imperceptible and recovery might take several minutes.

3. Electrical excitability of muscle

Since the flight muscle action potential in response to stimulation of cervical nerve is affected in some of the mutants at high temperature, the question arises whether muscle function itself is intact. The excitability of the flight muscles can be tested by direct injection of depolarising current. A longitudinal muscle fibre was impaled simultaneously with two KCl-filled micropipettes, one of which recorded the intracellular potential while the other was used to inject a 30 millisecond pulse of 200 to 600 nanoamperes of current, using bridge balance. The indirect response of the muscle to stimulation of the cervical nerve was monitored in the same preparation.

In normal flies, the response of the muscle to direct intracellular stimulation is a repetitive discharge of spike-like action potentials (Fig.) This response is somewhat variable; in many preparations the direct response persists unchanged on prolonged repetitive stimulation, while in others, repeated current injection reduces the excitability of the muscle. A majority of the preparations of normal flies remained excitable upto 39°C. At higher temperatures the response changed from sharp spike-like firing to a sinusoidal discharge and in about half of the preparations the spikes

became attenuated and were eventually lost irreversibly, presumably due to damage by high doses of current.

comatose^{ST-53}, para^{ts} and para^{ST-42} behaved at high temperatures essentially in the same manner as the wild type. Under conditions where the indirect response to cervical stimulation was lost at high temperature, the response to direct stimulation was not affected (Fig. and). In the mutants comatose and para therefore, the electrical excitability of the flight muscle itself is not responsible for paralysis.

In the case of shibire^{ST-139} on the other hand, the response to direct stimulation was greatly reduced along with the indirect response at temperatures above 30°C (Fig.). Electrical excitability of the muscle recovered on return to room temperature, although never fully. In shibire, therefore, there appears to be a temperature-sensitive lesion in the muscle itself.

4. Propagation of action potential in nerve

The propagation of action potential in nerves can be recorded extracellularly from a readily accessible nerve in the larva. Since, in these temperature-sensitive mutants, larvae as well as adults are paralysed at high temperature, larval nerves offer a convenient preparation for testing the effect of temperature on impulse conduction.

In the larva, eight pairs of abdominal nerves run from the ganglion to the abdominal segments (Fig.). Third instar larvae were dissected under Drosophila ringer and their nervous system exposed. One of the large nerves, the 7th or the 8th, was cut near the posterior end and sucked up in a micropipette with a tip diameter of 10 μmeter filled

with Drosophila ringer which served as the recording electrode. The stimulus was applied near the anterior end with a pair of ringer-filled micropipettes touching the nerve. With stimuli just above threshold, the response consisted of a single action potential of about 200 microvolts which persisted upon repetitive stimulation. As the stimulus amplitude was raised, a second, slightly delayed response was observed. The second response failed frequently when the repetition rate exceeded about 20 per second. It is likely that the first response was the result of direct stimulation of the nerve while the later response was indirect, resulting from antidromic excitation of the ganglion.

The nerves of normal larvae continued to fire above 39°C but comatose lost its response at 38°C. When brought back to room temperature, the impulses reappeared after a while (Fig.). Thus there is an effect of temperature directly on nerve excitation in this mutant. This is not the case with shibire^{ST-139} for the nerves of shibire larvae were indistinguishable from wild type, giving conducted action potentials at 39°C for several minutes. The larvae of the para group have not been examined so far.

5. The electroretinogram

The electroretinogram (ERG) of Drosophila, when evoked by a short flash of light, contains two major components, a corneal-positive spike and a negative wave (Hotta and Benzer, 1959). The negative wave is the primary response reflecting depolarisation of the photoreceptor cells. This triggers off the positive spike, which is believed to arise from the action potentials of the second order neurons in the lamina. The ERG of normal flies remains basically the same at temperatures up to 38°C, except for a contraction in the time scale. The same is true of

para^{ts}; heating the fly above its paralysing temperature does not change its ERG (Suzuki, et.al., 1971). In comatose, at 38°C the photoreceptor response remains intact, but the positive peak is lost. On returning to room temperature the ERG gradually recovers its normal form (Fig.). This effect of high temperature on the ERG is not as rapid as the paralysis of the motor system and is somewhat variable among individual flies as shown in Fig. . The same behaviour is shown by shibire^{ST-139} except that the positive peak is lost at a lower temperature of about 32°C and recovery is quicker, usually taking no more than one or two minutes. This is the same as reported by Hall (?) and Kelly (1974) for shibire. Hall has shown that, in genetically mosaic flies, it is the genotype of lamina and not the retina, that determines whether positive spike will be lost at high temperature.

Thus, in all three kinds of temperature-sensitive paralytic mutants, photoreceptor cell function is resistant to the temperature which causes paralysis. Synaptic transmission from the photoreceptors to the second order neurons in the lamina and excitability of the lamina must ramain intact in para, while in comatose and shibire, one or both of these processes break down at high temperature.

6. Temperature-induced firing of flight oscillator

The ensemble of indirect flight muscles in Drosophila and related dipterans is driven by neurons that fire in a characteristic pattern. The motor neurons innervating the different muscle fibers of a motor unit fire at close to the same frequency, maintaining fairly constant interspike intervals with stable phase relationships between different fibers of the unit. These firing patterns have been interpretted in

terms of a model which assumes that the neurons driving a motor unit share a common excitatory input and are mutually linked by lateral inhibitory connections. The part of the nervous system generating this patterned firing has been called the flight oscillator (Wilson, 1966; Wyman, 1966, 1969a, 1969b) Levine (1973) has analysed the flight oscillator of Drosophila.

While recording intracellularly from the dorsal longitudinal muscles of shibire^{ST-139} it was observed that, as the temperature approached 30°C, the muscles began to fire spontaneously in a manner reminiscent of natural flight. Fig. illustrates the constancy of the interspike interval and the phase-locking of two of the muscle fibers in a motor unit even thcugh the size of the action potential became greatly reduced as the temperature was raised. A pulse of cervical stimulation under these conditions produced a muscle action potential of the same reduced size. Temperature-induced firing of the flight oscillator could be maintained for many minutes in flies mounted in wax which did not beat their wings although the wings were free to move.

The above phenomenon did not occur in para, comatose or wild type flies. It appears that, in shibire^{ST-139}, elements that normally inhibit the flight oscillator are themselves inhibited at high temperature, so that the oscillator runs freely, providing input to the muscles. The latter respond with progressively lower action potentials as the temperature is raised. Incidentally, this response of the flight oscillator in shibire provides a convenient method for studying flight oscillations in solidly tethered flies unimpeded by the initiation of wing beats.

DISCUSSION

The nervous and the musclar systems of Drosophila are very complex and a given genetic lesion may affect various parts of the system to different degrees. Genetic alteration or loss of a specific membrane molecule, for instance, could, at one stroke. change the function of various neurons, synapses or muscles. As the system is wired up in a complex fashion, dysfunction of an inhibitory neuron might appear as an excitatory effect elsewhere. It is here that the possibility of constructing mosaic flies, with some parts mutant and the others normal, offers an incisive method for perturbing specific parts of the system in order to obtain information about its functional organisation.

In the para mutants the cessation of leg movements at high temperature is not due to the leg muscles themselves, since cervical stimulation at the paralysing temperature will still cause them to jerk. The flight muscles also remain excitable by direct intracellular stimulation. A clue to the site of the lesion is provided by the fact that, as the temperature is raised, the threshold for triggering the cervical nerve progressively increases. Even in this condition, once the signal reaches the muscle, a normal muscle action potential occurs. The para mutation thus appears to affect the excitability of the nerve.

The cervical connective contains over 3000 axons including a pair of giant fibers which attain a diameter of 10 µm (Hengstenberg,). These are likely to be the ones which are most readily stimulated and may be responsible for transmission of impulses to the legs and the flight muscles in these experiments. Electrophysiological and anatomical evidence

in favour of this assumption has been presented by Levine (1973). Perhaps the circuits which normally control spontaneous leg movement involve finer axons. These might be affected by mutation in the same way as the giant axons, but due to their smaller size, develop intractably high thresholds at a somewhat lower temperature. Thus, spontaneous leg movement would cease before cervically stimulated jerks, as the temperature is raised.

Alternative explanations are, however, possible. Suppose the effect of the para mutation is to block a certain class of synapses. Spontaneous leg movements, operating through one of these, will be blocked at the paralytic temperature. The fibers in the cervical connective might include some that control the legs via pathways involving synapses that are unaffected by the mutation. At elevated temperatures, a high stimulus voltage might trigger some of the alternative fibers which, perhaps due to their smaller size, are not triggered by lower stimuli.

The flight muscles of comatose remain excitable at the paralysing temperature, yet the action potential produced by cervical stimulation decreases in a graded manner. This graded decline of action potential resembles the effect of neuromuscular blocking agents on arthropod muscles. The fibrillar muscles of diptera are mutiterminally innervated (Tiegs, 1955). The muscle membrane is electrically excitable, as in vertiberate twitch fibers, but the excited response is not an "all or none" propogated action potential. The entire muscle fires at once giving a graded response whose size is proportional to initial depolarisation (McCann and Boettiger, 1961; Usherwood, 1969). Neuromuscular blocking agents such as tryptamine or ether produce a graded diminution of the response (Hill and Usherwood, 1961; McCann and Reece, 1966).

Our observations on comatose suggest that raising the temperature progressively eliminates individual junctions in a multiterminally innervated muscle. This would be expected if the neuromuscular junction itself or the finer terminals of the axons are inactivated by temperature. When the temperature is sufficiently high, all the neural input becomes blocked and no muscle spikes are produced by cervical stimulation even though flight muscle is still directly excitable. Failure of nerve conduction is demonstrable directly in the larval nerve, though the properties of the nerves in the larvae could, of course, be different from the adults. In the ERG the receptor potential remains, but the positive spike fades out, which can be understood in terms of a failure of action potentials in the second order neurons.

When the temperature is raised with shibire^{ST-139} the electrical excitability of the muscle membrane is clearly impaired. The fact that flight oscillator continues to function even though the muscle spike becomes greatly reduced shows that the nerve cells and their axons continue to function. The temperature-sensitive lesion, so far as the flight muscle is concerned, must therefore be postsynaptic. The failure of the leg response to cervical stimulation may also be due to the leg muscle although this is not proven. But this is clearly not the whole story for shibire. The setting off of the flight oscillator suggests neural dysfunction somewhere; so does the failure of the positive spike of the ERG, a neural defect which mosaic evidence indicates to be postsynaptic. Conduction in the larval nerve, on the other hand is not blocked in this mutant.

We have examined the locomotor behaviour of mosaic flies carrying different paralytic mutations. The results of these experiments will be

described elsewhere. One particular aspect of the paralysis of temperature-sensitive mosaic flies may, however, be briefly noted here. In comatose as in $para^{ts}$ and $para^{ST-42}$ individual legs behave as independent units during paralysis and may or may not be paralysed. In the case of $para^{ST-109}$ where paralysis requires a higher temperature, all six legs behave as a unit; in a given mosaic fly either all legs are paralysed together or none. Clearly in $para^{ST-109}$ a more central element in the neural network controlling leg movements is affected by the paralysing temperature. Using mapping methods described by Hotta and Benzer (1972) the embryological focus of this centre can be easily located. The temperature-sensitive mutants thus provide a convenient method for selective mapping of nerve centres.

These experiments represent an exploration into the possibility of using mutants to produce perturbations in various physiological parameters of the nervous system and the muscles. Each of the three mutant types examined has revealed a different physiological syndrome. Although the small size of Drosophila would at first sight appear to be prohibitive, it turns out that it is possible to make several kinds of electrophysiological measurements with relative ease. It seems not unreasonable to hope that the great genetic advantages of this organism will be usefully applied to neurophysiology.

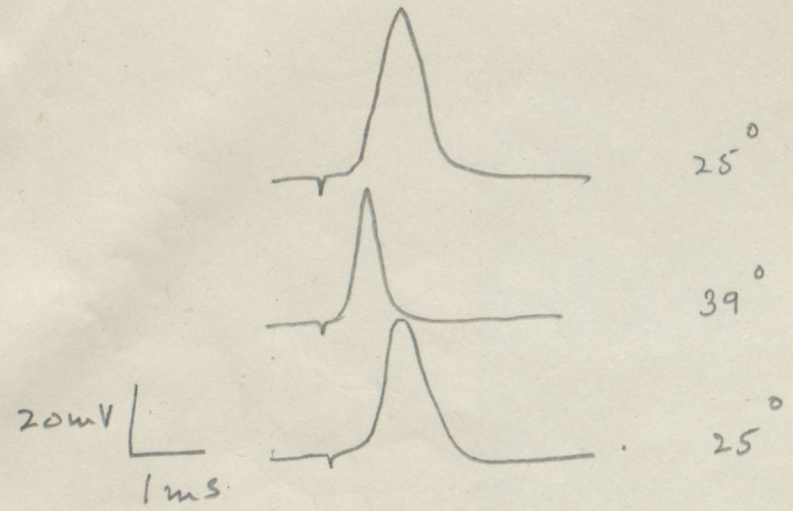


Fig 1. Effect of temperature on the response of wild type flight muscle. (CSQ #56)

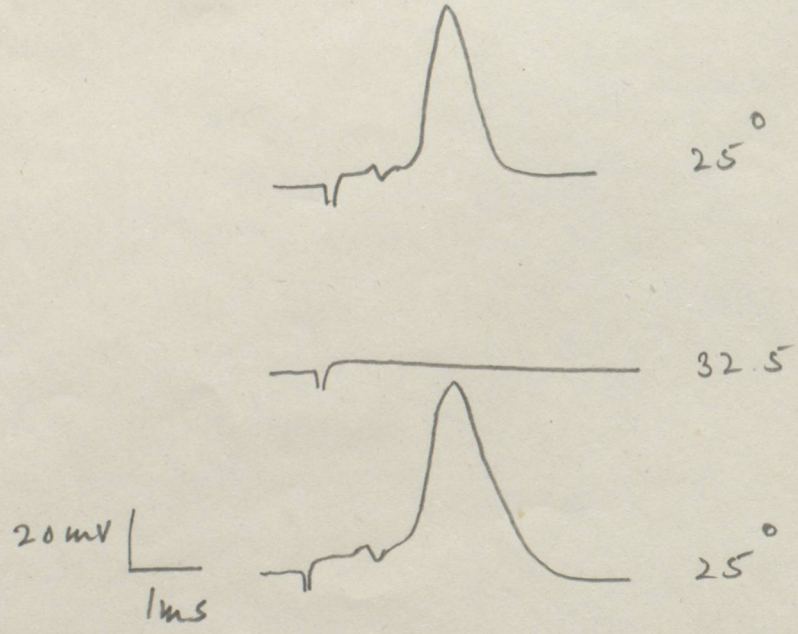


Fig. 2. All or none failure of muscle action potential in ST42 (#3a ♂). - Notice the disappearance of the pretympanic signal.

Paralysis Recovery.

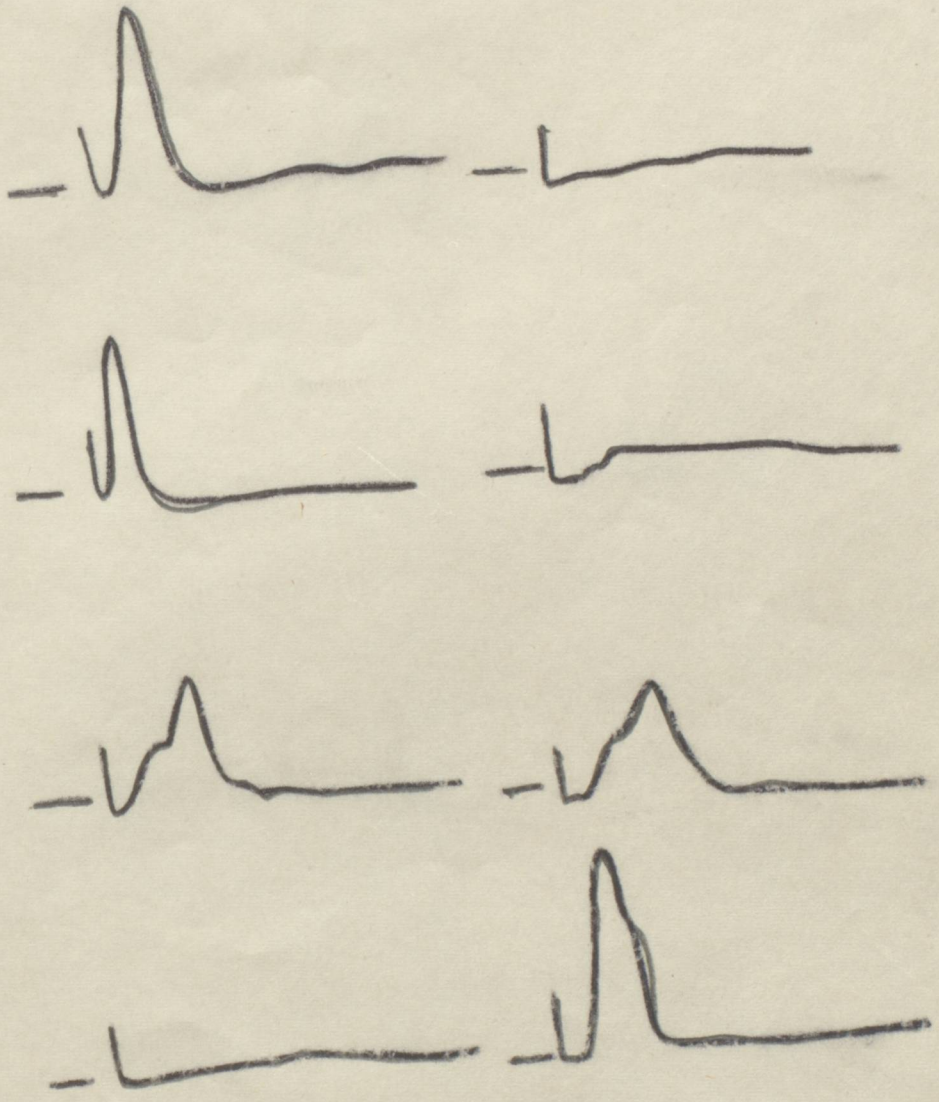


Fig 4. Effect of temperature on conduction.
(Also see the other record).

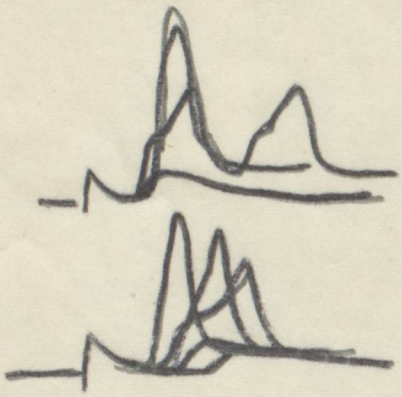


Fig 5. ST 139 paralysis + recovery

Fig 5. ST 139. Paralysis + recovery (135 # 5 + # 6).

Fig 6, 7, 8, 9 - Electrical excitability of CS, com, Hara + ST 139 -

(Perhaps a combined figure should be made).

Fig 10 + Larval Preparation —
Diagram in Horridge + Bullock.

Fig 11 + 12. Combine the larval
response of cs + com

25°
38°
25°

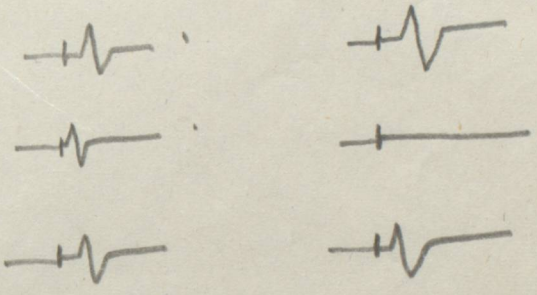
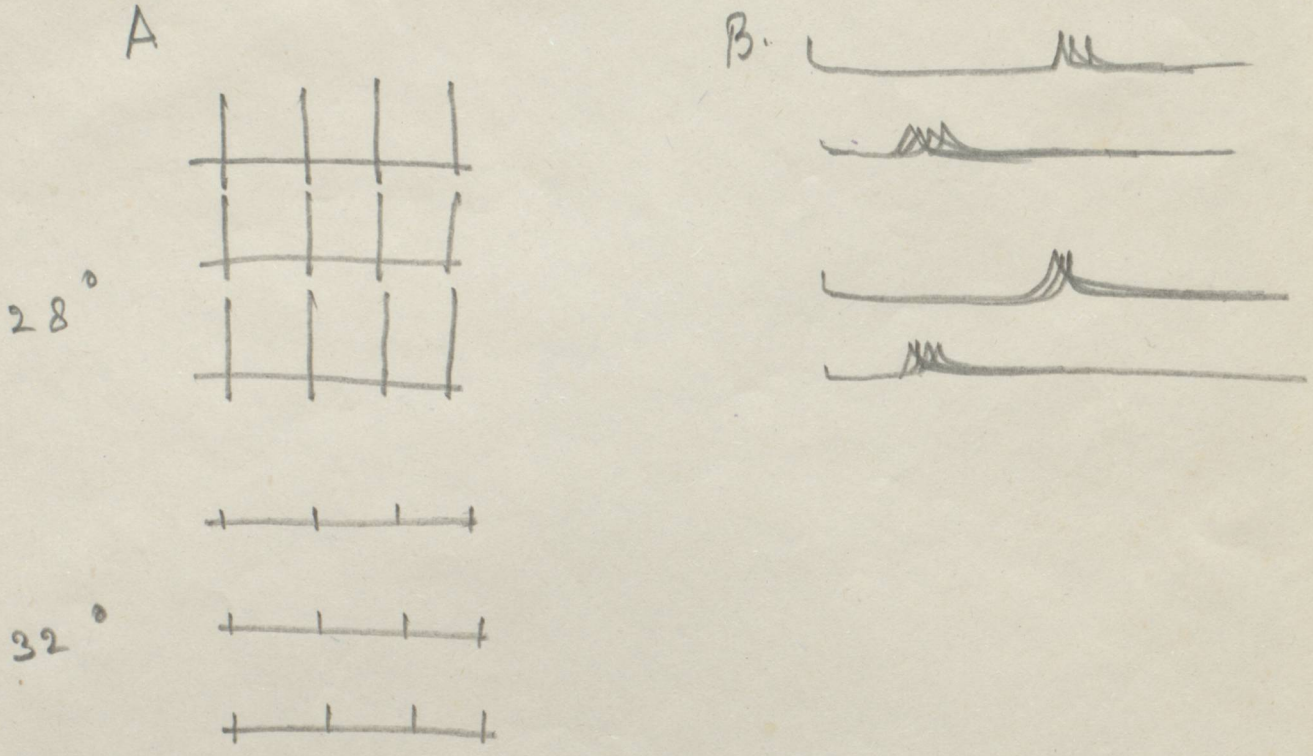


Fig 13. ERG Wild type + comatose

	cs	com
25		
38		
25		

Fig 14. Insect flight Oscillations

A - regular trains B. Phase locking of DLM # 3 + 5⁻.



New Scientist 8 May 1975

Of beads, chromatin and gene expression

At its first annual meeting last week in Heidelberg, the European Molecular Biology Organisation grappled with issues ranging from the structure of genes, to the way whole organisms put themselves together. This article reports on the molecular end of the spectrum. Next week a second article will discuss some aspects of developmental biology

Dr Roger Lewin
reports from Heidelberg

When molecular biologists had become at least some way confident in understanding genetic mechanisms in single-celled organisms (particularly the laboratory favourite Escherichia coli) they turned their attention to more complex organisms, only to find their progress much rougher going. But with the sudden flood of DNA structure within chromosomes, molecular biologists can claim a real advance in coming to grips with gene organisation in multi-cellular organisms, an essential step to a complete understanding of genetic expression.

The DNA strand (in the form of the famous double helix) is closely associated with several groups of proteins within the chromosome, the most important of which in structural terms are the histones. The histone proteins are chemically basic in nature, while the second major group of DNA-associated proteins are acidic. Over the past several years the general notion has developed that histones are for turning off gene expression, whereas the acid proteins are involved in gene activation. Overall, this may be true, but the fact that in any stretch of chromatin (that is DNA and associated proteins) there is an equal weight of DNA and histones points to a major structural role, a role that is not inconsistent with gene inactivation of course. Until 1973 the structure of chromatin was seen as basically a DNA thread with histones wrapped around it. That picture is now totally reversed.

The emerging concept of mammalian chromatin is a highly organised core of histone molecules, around which the DNA helix is coiled. This view of chromatin structure goes some way to explaining the way that a one centimeter DNA strand is packed into a one micrometre long chromosome, it suggests ways in which chromosome structure may be altered during the cell cycle, and it is consistent with the current slender evidence on the mechanics of gene transcription.

The new ideas on chromatin come from three sources: direct visualisation with electron microscopy; studies on histone interactions; and controlled fracture of the DNA helix using a number of different enzymes.

Ada and Donald Olins, at the Oak Ridge National Laboratory, US, were the first to produce persuasive pictures of chromatin in its "new guise". At the beginning of 1974 they published electron micrographs of chromatin strands which gave the impression of "particles on a string". The particles (they called them v bodies) were roughly spherical with a diameter of about 70 angstroms and were joined by a very short DNA string 15 angstroms wide. The v bodies have now been christened "nucleosomes" by a group of researchers in Strasbourg. The difference between the Olins' result and that of the Strasbourg group is the diameter of the bead, being put at 125 angstroms by the latter who published in April this year. By now there have been many estimates of the bead size, and the more popular figure is closer to 125 angstroms than the original 70.

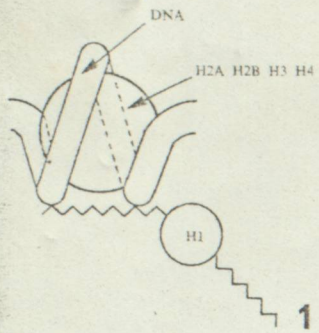
What are the beads made of? DNA and histones of course, but the arrangement of histone proteins appears to be far from random. Histones are divided into five classes according to their content of certain amino acids. Of the five, only four (H2A, H2B, H3 and H4) form part of the bead; the fifth (H1) is probably linked onto the outside of the bead.

Histones have a great affinity for one another, which is useful biologically, but awkward for the experimentalist, because they aggregate readily. By gentle separation techniques, Roger Kornberg and Jean Thomas at Cambridge, England, were able to tease the individual histones apart so as to get a good idea of how they were put together in the first place. In the middle of last year they reported that the core of the bead is based on a tetramer of two molecules each of H3 and H4 histones. Attached to this core are two molecules of H2A and H2B, probably one of each at each pole of the core. It is around this unit of eight protein molecules that the DNA helix is wrapped.

Conservative proteins

Unlike most proteins, histones show a very marked conservation of amino acid sequence. Usually, proteins that do the same job in different species vary considerably in their overall structure. Such variability is allowed, presumably, because the metabolic environment within which they operate differs between species, giving room for some individuality. The physico-chemical characteristics of DNA however are identical in all cells, from the simplest to the most complex. It is not surprising therefore that proteins whose job it is to interact with them should retain very similar structures. Of all the histones, it is the two that form the tetramer that show the strictest conservation, reflecting a *very* basic job in all forms of chromatin. Any inter-species differences in chromatin structure must derive from H2A and H2B variations, small though they are.

The discovery of the eight-histone component of the chromatin bead encouraged Kornberg in mid-1974 to propose a structure for the bead. Given that chromatin is composed of equal weights of DNA and histones, the amount of protein in the bead suggests a nucleic acid content of about 200 base pairs in the form of the helix. This length of



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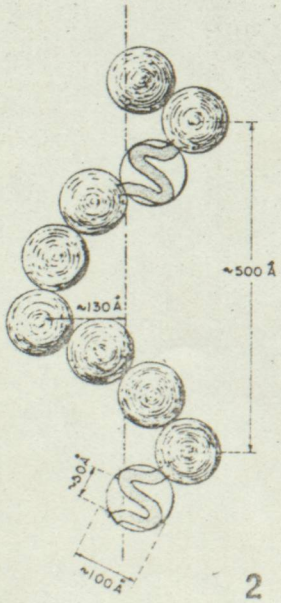
DNA amounts to about 670 angstroms, implying that the helix goes round the 100 angstrom diameter bead almost twice, given that it has to link bead to bead.

Morton Bradbury and his Biophysics Group at Portsmouth Polytechnic are also looking at the protein component of chromatin, using both X-ray diffraction and neutron scattering techniques. They show that the structure of histones is asymmetric, having discrete apolar and basic regions. Bradbury suggests that the apolar areas are involved in inter-histone associations, leaving the basic elements free to interact with the DNA. The availability of these basic regions to enzyme attack on the bead surface fits in very nicely with the ideas on chemical modification (such as methylation, acetylation and phosphorylation) of histones as part of the control of chromosome structure through the cell cycle. This applies particularly to H1 histone, phosphorylation of which might well cause contraction and cross-linking, thereby compacting the chromatin bead structure (see Figure 1).

For some time now a number of groups have been trying to get a direct count of the number of DNA base pairs in the nucleosome by snipping the inter-bead DNA thread with the enzyme DNase. A variety of enzyme sources have been used, giving a spectrum of answers, but until Marcus Noll (also at the Cambridge lab) developed a technique exploiting micrococcal nuclease, the solution remained remote: since then, as someone at the EMBO meeting remarked, "Everyone has been at it." And the answer is about 200 base pairs, fitting in with Kornberg's model.

The bead structure now has a lot of support, but it almost certainly represents only one level of chromatin superstructure; after all, the DNA packing ratio in the cell is close to 10 000:1. Using neutron scattering methods, Stanley Bram and his colleagues at the Institut Pasteur have collected information which suggests that the chromatin string of beads is further supercoiled with a radius of 130 angstroms and a pitch of 500 angstroms (see Figure 2). In their report earlier this year the Paris group suggested that "a further winding of the tertiary or quaternary structure . . . may wrap the coiled 100 angstrom unit fibril into a double chromatin helix to give [a] 250 angstrom diameter fibre". This kind of arrangement would increase the packing ratio considerably.



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How does this structure for chromatin relate to the issue of gene transcription? So far there is very little substantial evidence on this point. But some data suggest that replication of DNA goes in 200 base sequences (one bead's stretch of nucleic acid). Conceivably, transcription of DNA into RNA works on the same structural unit; the average structural gene (of 1200 base sequences) would span six beads.

Ever since a bead-like structure has been considered for chromatin, there has been the problem of how the DNA double helix can bend round the 100 angstrom diameter histone core: it just won't bend that far. Aaron Klug started to think about this problem at Cambridge, and was soon joined by Francis Crick. By playing with molecular models they came up with the idea that the DNA chain is kinked, possibly through as much as 98°. These kinks may well occur at every 10 base sequence, because extended digestion of chromatin with micrococcal nuclease produces DNA fragments 10 base pairs long. The kinked helix structure (which is not yet published) explains not only how the double helix might wrap itself round a protein core, but also presents a way by which exogenous sequence-specific molecules might gain access and recognise DNA base sequences as part of initiating or modifying transcription.

Evolution or junk!

One of the main issues of DNA transcription in eukaryotic cells is, what function does all that encoded information play? There is, after all, almost 1000 times more DNA in a eukaryotic (higher organism) cell as in a prokaryote (such as bacteria). Some must play a controlling role, but what of the rest? RNA synthesis in the cell nucleus produces a large amount of short-lived molecules, and only a small amount of messenger RNA that goes into the cytoplasm. Some people think that the nuclear RNA is a precursor of mRNA, but as yet there is no firm proof. The transcription and translation of genetic information in eukaryotes is much more sophisticated than in prokaryotes, so that molecular control equipment is to be expected somewhere. Nuclear RNA may indeed perform some role here. On the other hand, the long stretches of RNA that appear in the nucleus may simply be an unnecessary transcription of evolutionary potential, an RNA shadow of DNA sequences that may one day develop into real structural genes. The simplest explanation is that it is "junk"!

Junk or not, Eric Davidson, of Cal Tech, decided he would measure how much potential information there was in both mRNA and nuclear RNA in a sea urchin embryo containing just 600 cells. He reported in mid 1974 that the messengers contained information sufficient for 14 000 different protein species, some of which were produced abundantly (prevalent mRNA), while the rest (about 8 per cent) were in very short supply. Indeed, the limited production of the rare mRNA suggests that it is manufactured in small groups of cells, probably corresponding to the organisational pattern that is already apparent in the 600 cell embryo. In the nuclear RNA Davidson found genetic information sufficient for 140 000 proteins, a figure that is clearly ridiculous, but has to be explained.

The discovery of 14 000 different protein messages in sea urchin embryo mRNA suggests that throughout the further developmental and mature stages of the organism (and therefore other higher organisms) there will be a great many more structural genes expressed. This discovery will force people to reassess their notions of the number of structural genes expressible in higher organisms, particularly in man, for which a figure of 40 000 genes has been accepted until now.